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THE SYNTHESIS OF BUTYRIC AND CAPROIC ACIDS FROM ETHANOL AND ACETIC ACID BY CLOSTRIDIUM KLUYVERI

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Unpublished investigations in this laboratory have shown that *Clostridium kluyveri* can metabolize acetic acid and ethanol under anaerobic conditions producing butyric and caproic acids in accordance with equations. (1) and (2).

$$CH_3CH_2OH + CH_3COOH = CH_3CH_2CH_2COOH + H_2O \quad (1)$$

$$2CH_3CH_2OH + CH_3COOH = CH_3(CH_2)_4COOH + 2H_2O \quad (2)$$

The relative yields of the two acids are determined by the relative amounts of acetic acid and ethanol available. If acetic acid is present in excess, a considerable amount of butyric acid is formed, while if ethanol is in excess, caproic acid is the main product. These relations suggest that butyric acid may be an intermediate in the synthesis of caproic acid from acetic acid. This is supported by the observation that ethanol and butyric acid can be converted to caproic acid according to equation (3).

$$CH_3CH_2OH + CH_3CH_2CH_2COOH = CH_3(CH_2)_4COOH + H_2O \quad (3)$$

In the present investigation we have obtained positive proof for the conversion of acetic acid to caproic acid via butyric acid by studying the action of *Cl. kluyveri* on media containing synthetic fatty acids labeled with the long-lived radioactive carbon isotope C^{14}. In addition we have obtained various types of evidence which help to elucidate the mechanism of fatty acid synthesis from C_2 molecules.

Experimental Methods.—Carboxyl-labeled acetic, butyric and caproic acids were prepared from C^*O_2 and methyl iodide, propyl bromide and amyl bromide, respectively, by the Grignard synthesis.[1]

The general technique of estimating the C^{14} content of carbon dioxide and fatty acids has already been described elsewhere.[2] The barium salts

of the fatty acids dried at 100°C. were used in the radio-assay. The individual acids were separated from mixtures by the distillation procedure of Schicktanz, *et al.*[3] The purity of each acid was established by Duclaux distillation. The methods used in locating C^{14} in the individual carbon atoms of acetic and butyric acids have been described previously.[2, 4, 5] The occurrence of C^{14} in the carboxyl group of caproic acid was determined by decarboxylating the barium salt. Control experiments with synthetic caproic acid showed that this method yields 52 per cent of the carboxyl carbon as barium carbonate.

The bacteria were grown in media of the following composition in g. per 100 ml.: ethanol 0.3–0.8, sodium salt of labeled acetic or butyric acid 0.3–0.6, yeast autolysate 0.3, pH 7.2, phosphate buffer $M/40$, $(NH_4)_2SO_4$ 0.1, $MgSO_4 \cdot 7H_2O$ 0.01, $FeSO_4 \cdot 7H_2O$ 0.0001, sodium thioglycollate 0.05,

TABLE 1

THE FERMENTATION OF CH_3CH_2OH AND CH_3C^*OOH BY *Cl. kluyveri*

Experiment 1

COMPOUND	MM	CTS./MIN./MM	TOTAL CTS./MIN.
Before growth			
Ethanol	ca. 1.1	....	....
Acetic acid	0.830	18,900 ± 300	15,700
Carbon dioxide	ca. 0.2	....	....
After growth			
Acetic acid	0.395	7330 ± 150	2895
Butyric acid	0.196	18,200 ± 300	3570
Caproic acid	0.257	24,500 ± 500	6300
Carbon dioxide	0.185	26 ± 50	5
Ethanol	0.217	260 ± 50	74
Non-volatile cpds.	...	....	193
Percentage recovery of C^{14}			83

traces of salts of Ca, Mn and Mo, distilled water. In some experiments sodium carbonate (0.025 g. per 100 ml.) was added as a sterile solution after autoclaving the medium. Oxygen was removed by means of either a pyrogallol-potassium carbonate or an Oxsorbent seal. The cultures were incubated at 32–35° until growth ceased before being analyzed.

Results.—When *Cl. kluyveri* was allowed to grow in a medium containing approximately equivalent amounts of ethanol and synthetic carboxyl-labeled sodium acetate, radioactive butyric and caproic acids were formed (table 1). The isotope was proved to be present in these acids, rather than in some associated compound, by establishing the constancy of the specific activities of the barium salts prepared from different fractions of a Duclaux distillation (table 2). A little C^{14} was also found in ethanol and unidentified non-volatile compounds, but no significant amount was present in carbon dioxide. It should be noted that there is no net production of

carbon dioxide in this fermentation. The recovery of C^{14} in all forms after growth was about 83 per cent of that initially added as acetic acid.

A particularly significant fact revealed by this experiment is the decrease in the molar activity of acetic acid during the fermentation. At the beginning of the experiment the activity expressed in cts./min./mM was 18.9×10^3 while at the end the value was 7.3×10^3. The simplest and most likely explanation for this reduction in activity is the oxidation of ethanol to inactive acetic acid or some closely related compound in equilibrium with it. This would cause a dilution of the labeled acetic acid.

TABLE 2

SPECIFIC ACTIVITIES OF BARIUM SALTS OF BUTYRIC AND CAPROIC ACIDS PREPARED FROM DUCLAUX DISTILLATIONS

Experiment 1 (total volume = 110 ml.)

FRACTION	CTS./MIN./MG. OF Ba SALT
Butyric acid	
0–20 ml.	47.5
20–40 ml.	47.6
40–110 ml.	46.4
Caproic acid	
0–20 ml.	63.7
20–110 ml.	62.2

Another possible explanation for the decrease in activity of acetic acid is that it is in chemical and isotopic equilibrium with the higher fatty acids formed from it and ethanol. This was tested by two experiments in one of which butyric acid labeled in the carboxyl group was added at the beginning of the fermentation; in the second experiment butyric acid labeled on all four carbon atoms was used. In the experiment with carboxyl-labeled butyric acid (table 4), no C^{14} could be detected in the final acetic acid, thus indicating that no exchange occurred between the carboxyl groups of butyric acid and acetic acid. In the second experiment with completely labeled butyric acid, a little C^{14} was recovered in the final acetic acid but it was less than 9 per cent of the amount to be expected if butyric and acetic acids were in isotopic equilibrium, and even this small effect may have been due to a slight contamination of the original butyric acid by labeled acetic acid. An exchange of C^{14} between acetic acid and the higher fatty acids can therefore be excluded as an explanation for the observed decrease in activity of acetic acid.

A third possible explanation for this effect is an exchange of C^{14} between ethanol and acetic acid as a result of reactions involving acetaldehyde or a similar compound as an intermediate. If such a reaction occurs, the residual ethanol should have the same molar activity as the acetic acid. Actually, the activity of the alcohol is of a lower order of magnitude

(table 1). Therefore an exchange of C^{14} between acetic acid and ethanol is not consistent with the observed results.

Whatever the actual mechanism of the dilution of the active acetic acid, the magnitude of the effect is about what would be expected if all the ethanol is oxidized to acetic acid while the latter is being converted to higher fatty acids. To calculate the expected decrease of acetic acid activity on this basis, let us assume that (1) the fermentation proceeds in two successive steps represented by equations (1) and (3), and that during the fermentation all the ethanol is first converted to acetic acid or some compound in isotopic equilibrium with it. In step 1, the formation of butyric acid from ethanol and acetic acid, let x = the amount of C^{14} present in acetic acid at any time, x_0 = the initial C^{14}, x_1 = the C^{14} at the end of step 1, V = the amount of acetic acid present at any time, V_0 = the initial acetic acid, and V_f = the final acetic acid. Now during step 1, when a small amount (ΔV) of alcohol is oxidized to inactive acetic acid, the removal of active acetic acid by conversion to butyric acid will be equal to $2\Delta V$. The loss of C^{14} from acetic acid is therefore

$$-\Delta x = -2\Delta V/V - \Delta V \cdot x. \tag{4}$$

In the limit as ΔV approaches zero

$$dx/x = 2dV/V. \tag{4a}$$

Integrating between the limits of V_0 and V_f, and x_0 and x_1 and changing to $\log_{10}$ we get

$$\log x_1/x_0 = 2 \log V_f/V_0 \tag{5}$$

or

$$x_1 = x_0(V_f/V_0)^2. \tag{5a}$$

In step 2 (equation 3), the conversion of alcohol and butyric acid to caproic acid, there is no change in the total quantity of acetic acid; V_f is therefore a constant. C^{14} will nevertheless be lost from acetic acid if, as is assumed, it is an intermediate in the utilization of ethanol. When a small amount of alcohol (ΔA) is used, the loss of C^{14} from acetic acid is

$$-\Delta x = \Delta A/(V_f + \Delta A) \cdot x. \tag{6}$$

In the limit

$$-dx/x = dA/V_f. \tag{6a}$$

Integrating between the limits x_1 and x_f, and 0 and A_f, where A_f is the total amount of alcohol used in step 2

$$\ln x_1/x_f = 2.3 \log x_1/x_f = A_f/V_f \tag{7}$$

or

$$\log x_f = \log x_1 - A_f/2.3V_f \tag{7a}$$

When this method of calculation is applied to the data of table 1 where x_0 = 15,600 cts./min., V_0 = 0.830 mM, V_f = 0.395 mM and A_f = 0.257 mM, it is found that x_1 = 3540 cts./min., and x_f = 1840 cts./min. The calculated molar activity of acetic acid at the end of the fermentation is therefore 1840 cts./min./0.395 mM = 4660 cts./min./mM. This value is of the same order of magnitude though somewhat smaller than the observed molar activity of 7330 cts./min./mM. The discrepancy may be due to the fact that butyric and caproic acids are formed simultaneously rather than successively during part of the fermentation; this would raise the calculated activity of the final acetic acid and bring it into closer agreement with the observed value. The general conclusion to be drawn from a comparison of the calculated and observed decrease in the molar activity of acetic acid is that the results support the view that alcohol is oxidized to acetic acid or a closely related compound which is then condensed to butyric and caproic acids.

The following information was obtained concerning the distribution of C^{14} in the fatty acids at the end of the fermentation.

TABLE 3

DISTRIBUTION OF C^{14} IN BUTYRIC ACID

Experiment 2

CARBON ATOM	CTS./MIN./MM	PER CENT OF TOTAL RECOVERED
Carboxyl	1680 ± 50	54 ± 2
Alpha and gamma (average)	28 ± 50	1 ± 2
Beta	1395 ± 30	45 ± 2

The final like the initial acetic acid contained C^{14} only in the carboxyl group. The actual results were 7400 ± 200 cts./min./mM of carboxyl carbon and 0 ± 30 cts./min./mM of methyl carbon.

Data on the distribution of C^{14} in butyric acid are presented in table 3. The butyric acid was obtained from a second experiment identical with experiment 1. The data show that C^{14} is present in the carboxyl and beta positions but not in the alpha and gamma positions. The quantity of C^{14} in the carboxyl and beta carbon atoms is almost equal, though there appears to be a small but significantly greater amount in the carboxyl carbon. The smaller C^{14} content of the beta carbon may result from the fact that the beta (and gamma) carbon atoms are derived from an oxidation product of ethanol which is not in complete isotopic equilibrium with the preformed acetic acid.

No method was available for determining the complete distribution of C^{14} in caproic acid. The presence and amount of C^{14} in the carboxyl group could, however, be determined by decarboxylation of the barium salt. We found 4150 cts./min./mM in the barium carbonate obtained by the

decarboxylation of barium caproate containing 11,600 cts./min./mM. Therefore, 35.7 per cent or slightly more than one-third of the C^{14} was present in the carboxyl group. The data are consistent with the view that the C^{14} is equally distributed among three of the six carbon atoms (probably those in the carboxyl, beta and delta positions), but this is not certain.

The results of experiment 1 (table 1), considered in conjunction with other information now available, lead us to postulate that the following reactions may be involved in the formation of butyric and caproic acids from acetic acid and ethanol.

$$CH_3CH_2OH + K_2HPO_4 \rightarrow CH_3COOPO_3K_2 + 4H \quad (8)$$

$$CH_3COOPO_3K_2 + ADP \rightleftharpoons CH_3COOH + ATP \quad (9)$$

In the presence of synthetic CH_3C^*OOH, the forward reaction (9) would cause a dilution of the active acetic acid and the reverse reaction would introduce C^{14} into the carboxyl group of acetyl phosphate. Although there is at present no direct evidence to indicate the occurrence of acetyl phosphate in this fermentation, the compound is almost certainly formed by *Cl. butylicum*,[6] an organism which also produces butyric acid (and butyl alcohol) from acetic acid.[5] Moreover the chemical properties of acetyl phosphate are consistent with its being an intermediate in fatty acid synthesis.[7, 8] The labeled acetyl phosphate and the labeled acetic acid are assumed to condense to yield a product that is reduced to butyric acid. This reaction is not reversible.

$$CH_3C^*OOPO_3K_2 + CH_3C^*OOH + 4H \rightarrow CH_3C^*H_2CH_2C^*OOH + K_2HPO_4 + H_2O. \quad (10)$$

A similar type of condensation and reduction involving butyric acid and acetyl phosphate could lead to the formation of caproic acid labeled in the carboxyl, beta and delta positions.

$$CH_3C^*OOPO_3K_2 + CH_3C^*H_2CH_2C^*OOH + 4H \rightarrow CH_3C^*H_2CH_2C^*H_2CH_2C^*OOH + K_2HPO_4 + H_2O. \quad (11)$$

Reaction (11) represents only one of two possible ways in which caproic acid could be formed. Instead of there being a direct addition of the acetyl group to the terminal methyl group of butyric acid as is indicated above, the high energy phosphate group could be first transferred to butyric acid to give butyryl phosphate which could then condense with the methyl group of acetic acid.

$$CH_3C^*H_2CH_2C^*OOH + CH_3C^*OOPO_3K_2 \rightleftharpoons CH_3C^*H_2CH_2C^*OOPO_3K_2 + CH_3C^*OOH. \quad (12)$$

$$CH_3C^*H_2CH_2C^*OOPO_3K_2 + CH_3C^*OOH + 4H \rightarrow CH_3C^*H_2CH_2C^*H_2CH_2C^*OOH + K_2HPO_4 + H_2O. \quad (13)$$

Evidence has been presented by Koepsell, *et al.*,[6] that a transphosphorylation between acetylphosphate and butyric acid occurs under the influence of an enzyme preparation obtained from *Cl. butylicum*. With either mechanism the isotope distribution of the resulting caproic acid would be the same under the conditions of experiment 1.

It is possible, however, to distinguish between these two types of condensation by carrying out a fermentation of ordinary ethanol and synthetic butyric acid labeled in the carboxyl group. The position of the C^{14} in the resulting caproic acid must depend on the way in which the condensation occurs. This is illustrated in equations (14) and (15) where the inactive acetyl phosphate and acetic acid are assumed to originate from the oxida-

$$CH_3COOPO_3K_2 + CH_3CH_2CH_2C^*OOH + 4H \rightarrow CH_3CH_2CH_2CH_2CH_2C^*OOH + K_2HPO_4 + H_2O. \quad (14)$$

$$CH_3(CH_2)_2C^*OOPO_3K_2 + CH_3COOH + 4H \rightarrow CH_3(CH_2)_2C^*H_2CH_2COOH + K_2HPO_4 + H_2O. \quad (15)$$

tion of ethanol. It can be seen that a condensation of acetyl phosphate and labeled butyric acid would yield carboxyl-labeled caproic acid, while a condensation of labeled butyryl phosphate and ordinary acetic acid would yield caproic acid labeled in the beta position.

TABLE 4

THE FERMENTATION OF CH_3CH_2OH AND SYNTHETIC $CH_3CH_2CH_2C^*OOH$ BY *Cl. kluyveri*

Experiment 3

COMPOUND	MM/10 ML.	CTS./MIN./MM	TOTAL CTS./MIN.
Before growth			
Acetic acid	0.100	0	0
Butyric acid	0.384	17,200 ± 500	6610
Ethanol	ca. 1.7	0	0
After growth			
Acetic acid	0.101	0 ± 100	0
Butyric acid	0.05	18,000 ± 10,000	955
Caproic acid	0.427	12,600 ± 500	5400
Ethanol	0.306	250 ± 50	75

The results of an experiment designed to distinguish between these possible condensation mechanisms are given in table 4. Alcohol was added in excess to favor a high conversion of butyric acid to caproic acid.

It can be seen in table 4 that most (81.7 per cent) of the C^{14} originally present in butyric acid was converted into caproic acid. The molar activity of the caproic acid (12,600 ± 500 cts./min./mM) is somewhat less than that of the original butyric acid (17,200 ± 500 cts./min./mM) due to the fact that part of the caproic acid was completely built up from in-

active ethanol and acetic acid. This is evident from the fact that the quantity of caproic acid formed (0.427 mM) was greater than the butyric acid originally added (0.384 mM). When this dilution is taken into account, the calculated molar activity of the final caproic acid (17,200 cts./min./mM $\times$ 0.384 mM/0.427 = 13,400 cts./min./mM) agrees fairly well with its observed molar activity (12,600 cts./min./mM).

The caproic acid formed in this experiment contained little or no C^{14} in the carboxyl group. The barium carbonate obtained by decarboxylation from two samples of barium caproate showing activities of 845 $\pm$ 20 and 657 $\pm$ 15 cts./min., were found to give only 6 $\pm$ 3 and 5 $\pm$ 3 cts./min., respectively. It will be recalled that synthetic carboxyl-labeled barium caproate yields 52 per cent of its C^{14} as barium carbonate.

The absence of C^{14} from the carboxyl group of caproic acid proves that a condensation of the type shown in equations (11) and (14) does not occur. The results are, however, consistent with the mechanism shown in equations (13) and (15), involving a condensation of butyryl phosphate with the methyl group of acetic acid. The evidence in favor of this type of condensation would be stronger if the C^{14} had been proved to be present only in the beta position. This has not yet been accomplished, but further work on this problem is in progress.

There are a few other points concerning experiment 3 (table 4) that should be mentioned.

The complete absence of activity in acetic acid definitely proves that the conversion of acetic to butyric acid is not appreciably reversible at least so far as the carboxyl carbon atom is concerned. Evidence has already been presented above against any considerable exchange of carbon between acetic acid and the alpha, beta or gamma carbon atoms of butyric.

A small but definite amount of activity was found in the neutral volatile compounds, reported as ethanol. It is possible that this activity is actually present in ethanol, though this would be difficult to reconcile with the fact that there is no activity in acetic acid which seems to be an intermediate between butyric acid and ethanol. Another possibility is that the activity attributed to ethanol is actually present in an associated compound such as butyl or hexyl alcohol. Since the molar activities of the C_4 and C_6 compounds are relatively very high, a small (1–2 per cent) contamination of the ethanol would account for the observed activity. With the small amount of alcohol and the low activity involved in the present experiment, the contamination theory could not be proved or disproved. However, it may be mentioned in this connection that the characteristic odor of *Cl. kluyveri* cultures suggests the presence of a higher alcohol or ester.

Summary.—1. When *Cl. kluyveri* is grown anaerobically in a medium containing ordinary ethanol and synthetic acetic acid labeled in the carboxyl group with C^{14}, labeled butyric and caproic acids are formed.

2. The butyric acid so formed has C^{14} almost equally distributed between the carboxyl and beta positions. The alpha and gamma positions are inactive.

3. The caproic acid has one-third of its C^{14} in the carboxyl group; probably the beta and delta positions are also labeled, though this has not been proved.

4. No active carbon dioxide is formed from carboxyl-labeled acetic acid. This indicates that carbon dioxide is not an intermediate in these reactions.

5. The C^{14} content of the residual acetic acid is much lower than that of the initial acetic acid. This evidently results from the oxidation of ethanol to acetic acid or a related compound in isotopic equilibrium with it.

6. When *Cl. kluyveri* is grown with ordinary ethanol and synthetic carboxyl-labeled butyric acid, C^{14} is found in caproic acid but not in acetic acid.

7. The active caproic acid so formed contains almost no activity in its carboxyl group.

8. The above facts are consistent with the view that the formation of butyric acid involves a condensation between acetic acid or a reactive derivative thereof, such as acetylphosphate, formed by the anaerobic oxidation of ethanol, and a second molecule of acetic acid. The condensation product is then reduced to butyric acid. Caproic acid formation involves a condensation of the carboxyl group of butyric acid or some related C_4 compound, like butyrylphosphate, with the methyl group of acetic acid.

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THE DIFFERENTIAL EQUATION OF THE DISTRIBUTION OF GENE FREQUENCIES

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The first attempt to determine the mathematical form of the distribution of gene frequencies in populations was based on the setting up of differential equations for certain special cases (Fisher, 1922,[1] 1930[2]). A correction and extension of these results came from expression of the conditions in an integral equation (Wright, 1929,[3] 1931[4]). A general solution has since been obtained for fully stationary distributions by a third method (Wright, 1937,[5] 1938[6]). The case of uniform flux has been treated less generally (Wright, 1938,[6] 1942[7]). Dr. A. Kolmogorov[8] has recently been kind enough to send me a reprint of an important paper on this subject which was published in 1935 but which had not previously come to my attention. While the application is restricted to a particular stationary distribution, the method of approach points to a more systematic formulation than before.

The situation discussed by Kolmogorov is that of a large population, consisting of many subgroups of size n, each of which receives a certain number (k) of immigrants from the general population but otherwise breeds within itself. The average rate of change of the gene frequency of subgroups, in which p is the frequency of a given gene, is represented by $A = \Sigma(\Delta p) = (k/n)(\bar{p} - p)$ where $\bar{p}$ is the mean value of p in the whole population. The variance of p, due to accidents of sampling in one generation, is represented by $B = \Sigma(\Delta p)^2 = pq/2n$, $q = 1 - p$. It is stated, without demonstration, that the distribution $u(p)$ of gene frequencies among subgroups after a stationary state has been reached, answers to the differential equation

$$\frac{1}{2}\frac{\partial^2}{\partial p^2}(Bu) - \frac{\partial}{\partial p}(Au) = 0. \tag{1}$$

The pertinent solution is given as

$$u(p) = p^{4k\bar{p}-1}q^{4k\bar{q}-1}/B(4k\bar{p}, 4k\bar{q}). \tag{2}$$

The effect of selection in this situation is discussed briefly without, however, modifying $u(p)$ by introduction of the selection term, $\alpha p^2 q$, into A.

It is noted that the same formula (2) had previously been derived by the present author[4] by a different method. Equation (1) has, however, broader implications if valid for the general case $A = \Sigma(\Delta p)$, $B = \Sigma(\Delta p)^2$ and not merely for the particular case $A = (k/n)(\bar{p} - p)$, $B = pq/2n$.

The Immediate Factors of Evolutionary Change.—The immediate factors that tend to cause systematic changes (Δq) in gene frequency (q) may be listed exhaustively[4, 5] as (a) mutation pressure, $\Delta q = v(1 - q) - uq$, where v and u are the rates of mutation to and from the gene in question, (b) immigration pressure, $\Delta q = m(q_i - q)$ (Kolmogorov's A) where m is the proportion of replacement by immigrants and q_i is the gene frequency in these, and (c) selection pressure, which may take widely diverse forms but in the important case of constant relative selective values (W) for each multiple factor genotype in a random breeding population takes the form $\Delta q = q(1 - q)\frac{\partial \overline{W}}{\partial q}/r\overline{W}$, where r is 1 in haploids, 2 in diploids, the usual case, 1.5 for sex linked genes (if equal numbers of males and females), 4 in tetraploids, etc.[7, 9] In addition to these systematic pressures are (d) the random variations, δq, due to accidents of sampling, the variance of which is $\sigma^2_{\delta q} = q(1 - q)/rN$ in a population of effective size N (Kolmogorov's B). The diploid case ($r = 2$) will be assumed in what follows.

The Stationary Distribution of Gene Frequencies.—Systematic pressure toward the gene frequency, at which $\Delta q = 0$ and the cumulative effects of accidents of sampling determine a probability curve $\varphi(q)$ describing the frequencies which would be exhibited in the long run by the value of q for a particular gene in a population subject to constant conditions. This distribution may also be interpreted as that exhibited at one time by the values of q in a group of populations that are all subject to the same conditions (as in the case of Kolmogorov's $u(p)$). The deviations from the binomial square formula for genotypic frequencies in the total population, depend on the variance of $\varphi(q)$ under this interpretation.[4, 8, 10] In other cases $\varphi(q)$ may be used as the distribution at any time within either a class of non-allelic genes or an extensive series of multiple alleles,[11] all subject to the same conditions.

That equation (1) is, in fact, completely general for the stationary form of distribution may be shown by a slight modification of a method[5, 6] that has been used for derivation of $\varphi(q)$.

The conditions for stability of the distribution (including the terminal classes $q = 0$, $q = 1$) may be represented by two equations expressing the persistence of the mean and variance, respectively

$$\int_0^1 (q + \delta q + \Delta q)\varphi(q)dq = \int_0^1 q\varphi(q)dq. \qquad (3)$$

$$\int_0^1 (q - \bar{q} + \delta q + \Delta q)^2\varphi(q)dq = \int_0^1 (q - \bar{q})^2\varphi(q)dq. \qquad (4)$$

Noting that the mean value of δq is zero, and that δq is not correlated with q or Δq, these equations reduce to the following if the term in $(\Delta q)^2$ in (4) may be ignored. It may be noted in this connection that this term is negligible if Δq is of the same order as $\sigma^2_{\delta q}$ or less, while if of higher order,

systematic pressure dominates the results so completely that the distribution formula itself becomes unimportant.

$$\int_0^1 \Delta q\varphi(q)dq = 0. \tag{3a}$$

$$2\int_0^1 (q - \bar{q})\Delta q\varphi(q)dq + \int_0^1 \sigma_{\delta q}^2 \varphi(q)dq = 0. \tag{4a}$$

Putting $\Delta q\varphi(q)dq = d\chi(q)$ these conditions become

$$\chi(1) - \chi(0) = 0. \tag{3b}$$

$$2\int_0^1 \chi(q)dq - 2[\chi(1) + \bar{q}(\chi(1) - \chi(0))] - \int_0^1 \sigma_{\delta q}^2 \varphi(q)dq = 0. \tag{4b}$$

Substituting (3b) in (4b) the latter becomes

$$\int_0^1 [2\chi(q) - 2\chi(1) - \sigma_{\delta q}^2 \varphi(q)]dq = 0. \tag{4c}$$

A solution is obtained by removing the integral sign since the resulting equation not only satisfies (4c) but also (3b) (noting that $\sigma_{\delta q}^2 = 0$ if $q = 0$ or if $q = 1$, there being no sampling variance without alternatives in the sample).

$$\chi(q) - \chi(1) = {}^1/_2\sigma_{\delta q}^2 \varphi(q). \tag{5}$$

This can be solved for $\varphi(q)$ by differentiating the logarithm of the left-hand number and making the appropriate substitutions.[6]

$$\varphi(q) = (C/\sigma_{\delta q}^2)e^{2\int(\Delta q/\sigma_{\delta q}^2)dq} \tag{6}$$

where C is a constant such that $\int_0^1 \varphi(q)dq = 1$.

Since q increases by steps of $1/2N$ in a population of size N, the frequency of a given value of q is $f(q) = \varphi(q)/2N$. From a study[4] of simple cases ($N = 2$ or 3) in which the frequencies in the stationary state can be determined algebraically and from a more elaborate investigation by R. A. Fisher[2] of the subterminal region in certain cases, it appears that the frequencies are given with considerable accuracy by the formula except for the terminal classes, $q = 0$, $q = 1$. Consideration of the exchanges which occur between the terminal and neighboring classes leads[4] to the following approximate estimate for the terminal class, $q = 0$. That for $q = 1$ is analogous.

$$f(0) = f(1/2N)/4N[mq_1 + v]. \tag{7}$$

The differential equation for the completely stationary case is given by differentiation of (5). It comes under equation (1).

$$\frac{1}{2}\frac{d}{dq}(\sigma_{\delta q}^2 \varphi(q)) - \Delta q\varphi(q) = 0. \tag{8}$$

Since $\Delta q\varphi(q)$ is the proportion of the distribution which tends to be carried past a specified value of q by the systematic pressure Δq, the other

term must represent the net proportion which tends to be carried in the opposite direction by accidents of sampling in each generation.

The Case of Steady Flux.—There may be a practically stationary state of the proportions in all intermediate values of q in spite of steadily increasing frequency of one terminal class at the expense of the other, provided that the proportion lost by the donor terminal class is negligible. This cannot be the case if either mutation rate or immigration rate is appreciable, but may hold in the presence of strong selection pressure since selection pressure is nil in populations in which $q = 0$ or $q = 1$.

The differential equation for the case of steady flux must differ from (8) by a constant term (D), the net proportion of the total (excluding the recipient class) that is carried past each value of q in each generation.

$$\frac{1}{2}\frac{d}{dq}(\sigma^2_{\delta q}\varphi(q)) - \Delta q\varphi(q) + D = 0. \tag{9}$$

This is the general form given by one integration of (1) which is therefore the general differential equation for a steady state of the intermediate classes. It may be reduced to a linear equation of the first order by making the substitution, $y = \sigma^2_{\delta q}\varphi(q)$.

$$\frac{dy}{dq} - 2\left(\frac{\Delta q}{\sigma^2_q}\right)y + 2D = 0. \tag{10}$$

The solution for $\varphi(q)$ is as follows:

$$\varphi(q) = [e^{2\int(\Delta q/\sigma^2_{\delta q})dq}/\sigma^2_{\delta q}][C - 2D\int e^{-2\int(\Delta q/\sigma^2_{\delta q})dq}dq]. \tag{11}$$

The simplest special case is that in which Δq may be treated as zero (although there could be no flux if it were absolutely zero).

$$f(q) = \frac{C}{q(1-q)} - \frac{2D}{1-q}. \tag{12}$$

The case under (12) that is most important genetically is that of irreversible mutation at a rate so low that the donor class ($q = 0$, or $q = 1$) is not appreciably depleted. According to direction of mutation,

$$f(q) = 2v/q, \text{ or } f(q) = 2v/(1-q). \tag{13}$$

The ratio of the subterminal classes ($1/2N$ in this case) gives the probability that a single neutral mutation may reach fixation instead of elimination.

Returning to (12) the case in which $D = 0$ yields the corresponding simplest solution for a completely stationary state

$$\varphi(q) = 1/[2(0.577 + \log 2N)q(1-q)] \text{ (terminal classes excluded).} \tag{14}$$

The case in which there are constant relative selection coefficients for all genotypes ($\Delta q = q(1-q)\frac{\partial \overline{W}}{\partial q}/r\overline{W}$) gives an apparently simple but in general rather refractory form (assuming a given set of frequencies of other genes)

$$\varphi(q) = [\overline{W}^{2N}/\sigma^2_{\delta q}][C - 2D\int \overline{W}^{-2N}dq]. \qquad (15)$$

It will be convenient for later reference to cite the less general case $\Delta q = q(1-q)(s+tq)$, $\sigma^2_q = q(1-q)/2N$ which allows for any degree of dominance, provided s and t are both small.

$$f(q) = [e^{4Nsq+2Ntq^2}/q(1-q)][C - 2D\int e^{-(4Nsq+2Ntq^2)}dq]. \qquad (16)$$

Non-stationary States.—The general case, in which the proportion at each value of q is a function of time as well as of q itself, is given by the following, of which equation (1) is the case in which the left-hand member is zero. Time (T) is measured in generations.

$$\frac{\partial \varphi(q, T)}{\partial T} = \frac{1}{2}\frac{\partial^2}{\partial q^2}[\sigma^2_{\delta q}\varphi(q, T)] - \frac{\partial}{\partial q}[\Delta q\varphi(q, T)]. \qquad (17)$$

This can be reduced to an ordinary differential equation in the case in which the distribution has reached stability of form, with all classes (except the terminal ones) falling off at the same rate. Let $K = -\frac{1}{\varphi(q, T)}\frac{\partial \varphi(q, T)}{\partial T}$ be the rate of decay per generation.

$$\frac{1}{2}\frac{d^2}{dq^2}(\sigma^2_{\delta q}\varphi(q)) - \frac{d}{dq}(\Delta q\varphi(q)) + K\varphi(q) = 0. \qquad (18)$$

It may easily be verified that for the case in which fixation is occurring under the uncomplicated effect of inbreeding ($\Delta q = 0$, $K = 1/2N$) the only solution that does not involve negative frequencies is

$$\varphi(q) = 1, \quad \text{or } f(q, T) = C_0 e^{-T/2N}. \qquad (19)$$

In the case of irreversible mutation at an appreciable rate, $\Delta q = v(1-q)$ the rate of decay is easily shown to be $K = v$. Equation (18) is satisfied by the following value, originally derived by a different method.

$$f(q) = 2vq^{4Ns-1}. \qquad (20)$$

An analogous solution applies to the effect of swamping by immigration from a population in which the gene in question is fixed ($\Delta q = m(1-q)$, $K = m$)

$$f(q) = 2mq^{4Nm-1}. \qquad (21)$$

Comparison with Results by Other Methods.—The first attempt at determining the distribution of gene frequencies was made by R. A. Fisher[1] who arrived at differential equations for certain special cases, in terms, however, of a different variable than gene frequency, $\vartheta = \cos^{-1}(1 - 2q)$, used in order to make the sampling variance constant. A discrepancy between the rate of decay ($K = 1/4N$), derived by him for the case in which $\Delta q = 0$, and the value, $1/2N$, given by a general method[12, 13] for determining the rate of fixation of genes under any system of mating, led the present author[3, 4] to a different approach. The condition for a stationary state of the intermediate classes except for possible decay at rate K, was represented by the following equation in which q and x are recipient and donor classes, respectively, in the exchanges which occur from one generation to the next.

$$(1 - K)\frac{\varphi(q)}{2N} =$$

$$\frac{(2N)!}{(2Nq)![2N(1-q)]!}\int_0^1 (x + \Delta x)^{2Nq}(1 - x - \Delta x)^{2N(1-q)}\varphi(x)dx. \quad (22)$$

It could easily be seen that if $\Delta x = 0$, the equation is satisfied by $\varphi(q) = \varphi(x) = 1$, $K = 1/(2N + 1)$, the latter at least a close approximation to the rate of decay expected in this case. For the simplest stationary state, $K = 0$, $\Delta q \doteq 0$, the expression $\varphi(q) = Aq^{-1} + B(1 - q)^{-1}$ is indicated (cf. 12). Approximate solutions could also readily be obtained for the linear pressures of mutation and migration. Selection presented more difficulty.

On inspection of these results in manuscript, Fisher[2] was able to correct and extend his equations to obtain the following:

I. Case of uniform decay ($\Delta q = 0$)

$$\frac{\partial y}{\partial T} = \frac{1}{4n}\left[\frac{\partial^2 y}{\partial \vartheta^2} + \frac{\partial}{\partial \vartheta}(y \cot \vartheta)\right]. \quad (23)$$

$$y = A_0 e^{-T/2n} \sin \vartheta \quad \text{(cf. (19))}. \quad (24)$$

II. Stationary state, no selection ($\Delta q \doteq 0$)

$$\frac{dy}{d\vartheta} + y \cot \vartheta = -4nB. \quad (25)$$

$$y = A \operatorname{cosec} \vartheta + 4nB \cot \vartheta \quad \text{(general, cf. (12))}. \quad (26)$$

$$y = A \operatorname{cosec} \vartheta \quad \text{(symmetrical case, cf. (14))}. \quad (27)$$

$$y = 4nB(\operatorname{cosec} \vartheta + \cot \vartheta) \quad \text{(unidirectional mutation, cf. (13))}. \quad (28)$$

III. Stationary state, selection, no dominance, $\Delta q = aq(1 - q)$

$$\frac{dy}{d\vartheta} - (2an \sin \vartheta - \cot \vartheta)y = -4anA. \quad (29)$$

$$y = \operatorname{cosec} \vartheta(2A + Be^{-2an \cos \vartheta}) \quad \text{(general, cf. (16), } t = 0). \quad (30)$$

$$y = 4 \operatorname{cosec} \vartheta \frac{(1 - e^{-2an(1+\cos \vartheta)})}{(1 - e^{-4an})} \quad \text{(unidirectional mutation).} \quad (31)$$

In cases I and II, the results agreed with those obtained from the integral equation (22), as may be seen by making the substitutions $\cos \vartheta = 1 - 2q$, $yd\vartheta = \varphi(q)dq$, $d\vartheta/dq = 1/\sqrt{q(1 - q)}$. In case III it was the author's turn to make a correction in the selection term (published first[3] as e^{2Nsq}), by taking cognizance of a series of small terms erroneously thought to be negligible but which actually doubled the exponent. With this correction, there was agreement.[4]

The most general result[5] obtained for the completely stationary state by solution of (22) took into account all of the factors of change in the form $\Delta q = v(1 - q) - uq - m(q - q_i) + q(1 - q)(s + tq)$, $\sigma^2_{\delta q} = q(1 - q)/2N$.

$$\varphi(q) = Ce^{4Nsq+2Ntq^2} q^{4N(mq_i+v)-1}(1 - q)^{4N[m(1-q_i)+u]-1}. \quad (32)$$

This agrees with that obtained by substituting these values of Δq and of $\sigma^2_{\delta q}$ in (6).

The most general result[5,6] obtained by this method for the case of steady flux was for $\Delta q = q(1 - q)(s + tq)$.

$$f(q) = [e^{4Nsq+2Ntq^2}/q(1 - q)]\,[C - 2Dqe^{-(2Nsq+Ntq^2}\psi(2Nsq, 2Ntq^2)]. \quad (33)$$

where

$$\psi(a, 0) = 1 + \frac{a^2}{3!} + \frac{a^4}{5!} + \frac{a^6}{7!} \ldots = (e^a - e^{-a})/2a$$

$$\psi(0, b) = 1 + \frac{b}{3!} + \frac{7b^2}{5!} + \frac{27b^3}{7!} \ldots E_m b^m$$

$$E_m = (E_{m-1} + E_{m-2})/2m(m + 1).$$

No recurrence formula was recognized for the joint terms, $\psi(2Nsq, 2Ntq^2)$ but the coefficients were calculated[7] up to those pertaining to q^9.

The probability of fixation of a single mutation ($C = 2v$, $D = ve^{2Ns+Nt}/\psi(2Ns, 2Nt)$ for irreversible mutations from class $q = 0$, or $C = 0$, $D = -ve^{-(2Ns+Nt)}/\psi(2Ns, 2Nt)$ for irreversible mutations from class $q = 1$), could be calculated from the ratios of the subterminal classes, (Prob. $= \sqrt{s/2N}$ for a recessive mutation with selective advantage s, Prob. $= 2s$ for a dominant mutation with selective advantage s, or for a semidominant

with selective advantage s in the heterozygote). The last agrees with Fisher's conclusion.[2] Equation (33), with $t = 0$, is indeed equivalent to (31).

Comparison of (33) with (16) shows that if the former is correct, the following must hold:

$$\psi(2Nsq, 2Ntq^2) = \{e^{2Nsq+Ntq^2}/q\} \int e^{-(4Nsq+2Ntq^2)} dq. \qquad (34)$$

This was tested by expanding the two exponentials in (34), integrating each term of the second one and combining. The coefficients were in all cases identical with those published[7] for $\psi(2Nsq, 2Ntq^2)$.

Equation (22) also gave the solution (20) for the case of uniform decay under an appreciable mutation rate.[4]

The integral equation (22) and the differential equation (18) are clearly equivalent to a close approximation. They are not exact mathematical equivalents, however, as may be seen from the fact that K must be put $1/(2N + 1)$ in (22) if $\Delta x = 0$ to give the solution $\varphi(q) = 1$, while it takes its true value $1/2N$ in (18) to give the same result. In the other cases (except (12)) second order terms have been omitted in the series, obtained as solutions of the integral equation, which do not appear in the solutions of the differential equation. Neither equation, of course, represents the natural conditions exactly since integration is substituted for summation and differentials for minimal steps ($1/2N$) in gene frequency.

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EFFECT OF SMOKING ON TASTE THRESHOLDS FOR PHENYL-THIO-CARBAMIDE (PTC)

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In the course of experiments on tasting ability the question arose as to whether a person's threshold was the true one if he had been recently smoking. Salmon and Blakeslee (1935) tested a large group for PTC threshold and recorded the type of smoking for each individual (heavy, moderate, none). These records show that the position of the subject's threshold on the PTC scale is not correlated with his smoking habits. That is, a heavy smoker is just as likely to have a low threshold as a high one and vice versa. The amount that an individual might vary from his resting threshold when he smoked again had not been tested. (Resting threshold is here used as the value obtained after nine or more hours of abstinence from tobacco.)

Such a series of tests was therefore undertaken to show the effect, if any, that tobacco has on the taste apparatus, and to determine how long a time must elapse between smoking and a return to the original threshold.

In a search of the literature for the effect of tobacco on the various senses it was found that the following reactions have been studied: visual acuity, skin pressure sense, eye accommodation, skin reaction to slight electric currents, taste preferences, and mental and physical efficiency. In addition a number of workers have analyzed smoke and the tobacco in its various forms, snuff, pipe cuts, and cigarettes, for the ingredient causing the reactions. Bogen (1936) after careful analyses reports that no single explanation applies to the problem of irritation by tobacco smoke. The innocent bystander receives the sidestream rich in ammonia and other alkaline substances quite irritating to the eye and nasal passages. The smoker who gets the main stream through the cigarette or pipe stem has a more acid material thereby receiving his nicotine (alkaloid) as salts which are less irritating. The smoker does get heat, pyridine, volatile acids, tarry and phenolic constituents, and the aldehydes, furfural and acrolein. These all lead to irritation of the membranes. All workers are fairly well agreed that nicotine in whatever form absorbed is the principal toxic agent wherever the nervous system is involved.

Carefully controlled tests on mental and physical efficiency before and after smoking show a definite correlation between change in efficiency and smoking. Hull (1924) points out the following facts:

1. There is a large and uniform increase in the tremor of the hand lasting an hour and 23 minutes.
2. There is marked and uniform stimulation of the heart which is still present an hour and 40 minutes after smoking.
3. There is a minute increase in the speed of reading reaction time.
4. There is a gain in the rate of complex mental addition lasting an hour and 15 minutes but with no measurable effect on accuracy.
5. There is a high probability of loss in auditory memory and efficiency in rote learning, with recovery in an hour.

In a series of ten mental tests Bush (1914) reports a 10.5% decrease in efficiency after smoking.

Accommodation time, both near-to-far and far-to-near sight, has been studied over a period of years at Wellesley College. Homewood and Howe (1937) have shown that accommodation time is definitely decreased during the first 20 minutes after smoking, but increased from 40 to 60 minutes after. This agrees with Schrumpf-Pierron's (1927) statement that the effect of tobacco on the nervous system is first a stimulation and then a depression. They also found that the occasional smoker was stimulated more than the habitual one and depressed sooner and to a greater extent.

Wenusch and Schöller (1936) working on skin pressure found that sensitivity to both hair pressure and pendulum stroke was changed during smoking. They do not record any stimulating effect of smoking, only a depressant one.

From a survey of the literature on the changes in mental and physical states after smoking, and from a series of electrical tests on the finger before and after smoking, Mendenhall (1930) concludes that tobacco has the same effect as rest. If a person is tired and depressed, smoking will stimulate and bring him back to normal, if overexcited it will quiet him by its depressing action. If he is in a well-rested state it has no marked effect.

Sinnot and Rauth (1937) found the thresholds for sugar and salt high in smokers, but during a period of several days during which six smokers had abstained, their thresholds fell to the level of non-smokers. Laird (1939) tested the effect of the smoking habit on the preference for sweet or tart pineapple juice. He found no difference in the taste preferences of smokers and non-smokers among men, at any age, nor among women up to forty years. Among the women of fifty to sixty-eight years the non-smokers were like the other groups, but the smokers were predominantly in favor of the tart juice rather than the sweet. He did not test each individual before and after smoking, nor does he deal with threshold ability.

In testing the present group of subjects for the effect of smoking on tasting ability each person was his own control. Smokers were tested

only after nine or more hours of abstinence from tobacco. Of the 60 subjects, 32 were from the staff at Cold Spring Harbor and 28 were advanced students and staff at Wellesley College; 32 were habitual smokers and 28 non-smokers; 24 were men and 36 women. Concentrations of PTC were used from 1:5120 M up to 1:312.5 using a factor of two, such that each solution used in a test was twice as concentrated as that previously administered. (M in these numbers represents 1000.) In each test approximately 0.6 cc. was given by means of the straw method.

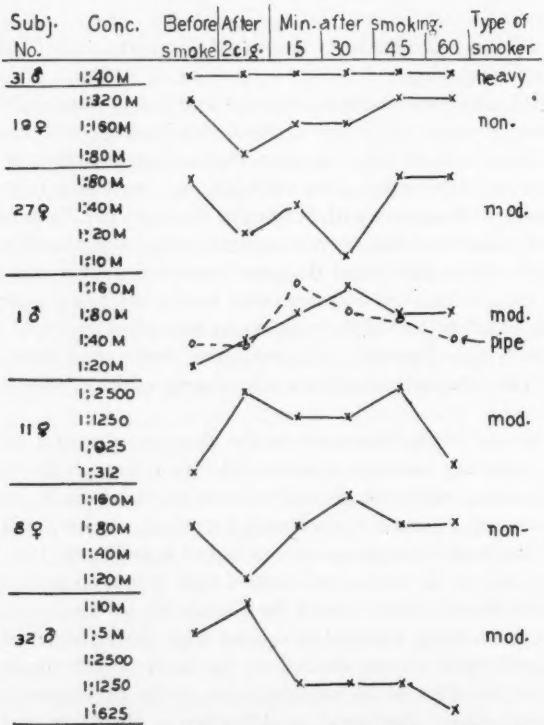

FIG. 1. ABILITY TO TASTE P.T.C. AFTER SMOKING.

Two types of experiments were performed. The procedure for the first series of tests was as follows:

1. The PTC threshold was determined.
2. The subject smoked two cigarettes of a standard brand in 10–15 minutes.
3. The PTC threshold was determined immediately after smoking and

at 15-minute intervals for one to two hours. (In several cases the tests ran still longer.) In the second series 10 individuals of the first series, both smokers and non-smokers, were tested as above except that the smoke was drawn through a dry flask for cooling and into the nose through a sterilized nose-piece keeping the mouth closed so that the smoke did not touch the taste buds.

A summary of the results of the first series may be found in table 1. Figure 1 shows typical PTC curves for each of the behavior groups.

TABLE 1

SUMMARY OF THE REACTIONS TOWARD PHENYL-THIO-CARBAMIDE AFTER SMOKING (SMOKE TAKEN IN THROUGH THE MOUTH)

CHANGE IN TASTING ABILITY	INDIV. IN GROUP	% OF TOTAL TESTED	NO. PTC GRADES INC.	NO. PTC GRADES DEC.	RETURN TO INITIAL LEVEL NO.	RETURN TO INITIAL LEVEL TIME	SMOKER YES	SMOKER NO	SEX ♂	SEX ♀
A. None	4	6.6	...	...	4	No change	2	2	1	3
B. Decreased only	32	53.3	...	1–5	22	30′–60′	15	17	12	21
					10	Over 60′				
C. Increased only	10	16.7	1–4	...	6	15′–60′	8	2	5	5
					4	Over 60′				
D. Decreased then increased	12	20.0	1–2	1–2	7	30′–60′	5	7	5	7
					5	Over 50′				
E. Increased then decreased	2	3.3	1	1–2	2	Over 60′	2	..	1	1
Totals	60	100			35	15′–60′	32	28	24	36
					21	Over 60′				

There were three types of reaction:

1. No change in tasting ability, group A (6.6%).
2. Decrease in tasting ability, groups B and D (73.3%): group B returned to initial level (53.3%); group D returned to initial level and then showed an increase (20%).
3. Increase in testing ability, groups C and E (20%): group C returned to initial level (16.7%); group E returned to initial level and then showed a decrease (3.3%).

These figures check very well with those of Mendenhall (1930) working on sensitivity to electric shock. He found that when tested before and after smoking (750 observations) 72.2% of the cases showed a depression while 28.3% showed a stimulation. In our work for taste 73.3% were depressed, 20% were stimulated and 6.6% showed no change.

Considering the time at which depression and stimulation occur the following summary may be made:

Time at which tasting ability is first lowered (groups B, D):

38 during smoking (10–15 minutes)
4 by 15 minutes after smoking
2 by 30 minutes after smoking

Time at which tasting ability is first increased (groups C, E):

8 during smoking
3 by 15 minutes after smoking
1 by 30 minutes after smoking

Time of greatest increase after an initial decrease (groups B, D):

5 by 15 minutes after smoking
10 by 30 minutes after smoking
16 by 45 minutes after smoking
10 by 60 minutes after smoking
3 not increased again by 60 minutes (one was still low at 105 min.)

Thus it may be seen that the initial depression occurs during smoking or by 15 minutes after. For those who show no initial decrease, stimulation occurs for most individuals during smoking or in 15–30 minutes after. If there is an initial decrease the return to the original threshold or to a period of stimulation is for most subjects at 30–45 minutes after smoking.

The taste apparatus according to Ranson (1939) is composed of taste buds on the tongue from which two sets of special afferent visceral fibers pass to the tractus solitarius via the chorda tympani (of the seventh cranial nerve) and the glosso-pharyngeal nerve. These taste fibers connect with the anterior part of the nucleus of the tractus solitarius. Further connections are rather vague but certainly reflexes to the gustatory center and the motor nuclei for mastication and swallowing exist. A cerebral center is not definitely fixed but a spot near the anterior end of the temporal lobe is thought to have such function. There are at least three synapses then between the surface of the tongue and consciousness.

According to the work done on other sensory-motor arcs the expected curve for taste should show an initial level, a more sensitive period, and then a depression period. But we have here an added factor to complicate results, namely, the exposure of the sensory endings, the taste buds, to the various drugs resulting from the combustion. It may be that the depressant effect on the taste buds antedates or coincides with the stimulative effect on the nerve cells and thus we get several forms of curves for the first half hour depending on the comparative strength of the two factors. Some individuals keep to the same level at first with later depression, others have a sharp early depression followed by stimulation, while others are stimulated immediately and to a greater degree than the depression

of the taste buds. We felt that it might be enlightening so to conduct the smoking period that the taste buds would not be touched by the smoke. Of the 10 individuals so tested 3 showed no change in tasting ability for 30 minutes to an hour followed then by a depression. Six had an initial stimulation followed at 30 to 45 minutes by a depression. These persons had all shown initial depression when smoking by mouth. One subject (of group C) who showed only stimulation when smoking by mouth had the same type of reaction (stimulation only) when smoking by "nose." Figure 2 shows the mouth and "nose" curves for two of these subjects.

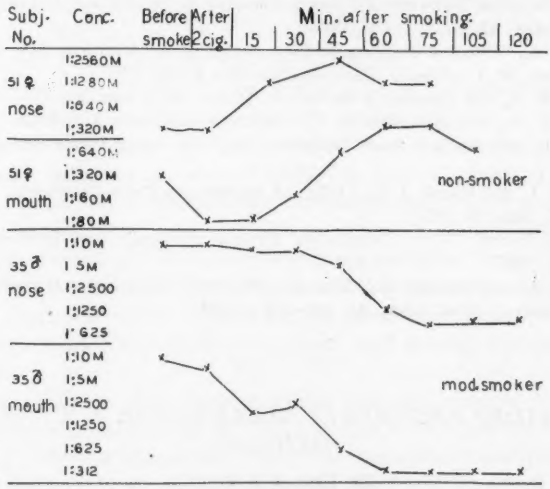

FIG. 2. EFFECT OF SMOKING BY NOSE.

It would seem then that the sharp depression noted for the larger number of subjects in series 1 may be due to the direct action of some product of combustion on the taste buds rather than to the effect of nicotine on the nerve cells.

We may therefore make the following conclusions:

1. There is a definite effect on the individual's ability to taste PTC after smoking, 73.3% of the subjects requiring stronger solutions in order to taste after smoking and 20% of them tasting weaker solutions.

2. The time which must elapse after smoking before the individual is a proper subject for tasting experiments varies with the individual. In the present series of tests only 58% had returned to the resting threshold within an hour, while some took several hours for recovery.

3. The initial effect of smoking in the larger number of cases is a direct dulling of the taste buds by some product of the combustion.

4. The true effect of nicotine on the nerve apparatus for taste appears to be the same as for other nerves tested—an initial stimulation with later depression.

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RESPIRATORY ENZYMES IN PARAMECIUM: 1. CYTOCHROME OXIDASE

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It has been generally stated, as the result of the work of a number of investigators,[1, 2, 3] that cyanide is without effect on the respiratory activity of Paramecium. The report of Kalmus[4] that respiration of Paramecium is depressed by cyanide has been largely disregarded because of serious defects in his experimental technique (cf. Howland and Bernstein[5]). Cyanide was likewise shown to be without effect on the respiration of a number of other ciliates,[6, 7, 8] and the conclusion was reached, as summarized by Lwoff,[8] that "Cette insensibilité à HCN et à CO n'est pas générale chez les Protozoaires, mais est jusqu'ici particulière aux Infusoires." The repeated failure to obtain depression of respiration in ciliates with cyanide and similar inhibitors led naturally to the belief that oxidations in these forms were mediated by a mechanism which was different from that of most aerobic cells.[9]

More recently, a reinvestigation of the effect of cyanide on the respiration of certain ciliates has been made. In these studies a number of technical improvements, not alone in the use of cyanide but also in rearing the animals and preparing them for respiratory measurements, were employed. Thus Baker and Baumberger[10] and Hall[11] showed that respiration in *Tetrahymena geleii* and *Colpidium campylum* could be inhibited by cyanide. Kitching,[12] in a study of contractile vacuole activity, obtained indirect evidence for cyanide susceptibility in the peritrich, Zoothamnion. Finally, the respiration of *Paramecium calkinsi* was shown to be inhibited to approximately 50 per cent of the normal by cyanide (Boell[13] and recently confirmed by Pace[14]) and by azide of the same concentrations as those usually employed for respiratory studies.

Since these inhibitors have been widely used to indicate the operation of the so-called cytochrome-cytochrome oxidase system in normal respiration, the conclusion was drawn[13] that in so far as susceptibility to cyanide and azide can be used as tests, the results suggest that the respiratory mechanism of Paramecium resembles that of most aerobic plant and animal cells. The purpose of this paper is to provide further support for the above conclusion by presenting direct evidence for the occurrence in Paramecium of an oxidative enzyme which presumably is cytochrome oxidase. Apparently this represents the first time that an oxidative enzyme has been obtained from a ciliate and studied by quantitative techniques.

Technique.—Respiratory measurements and cytochrome oxidase determinations were carried out in the Cartesian diver apparatus on known numbers of *Paramecium calkinsi* which had been reared and prepared for study by methods previously described by Boell and Woodruff.[15] In the cytochrome oxidase determinations, highly concentrated suspensions of well-washed Paramecium were used. Duplicate 10-cu. mm. samples were withdrawn in order to obtain a count of the number of animals present in a given volume of medium, and the remainder of the suspension (approximately 0.1 cc.) was ground into a cell-free brei by means of a motor-driven homogenizer. Appropriate amounts of the enzyme preparation were then added to measured quantities of cytochrome-c, ascorbic acid (Merck's Cebione) which had been neutralized with NaOH, and phosphate buffer at pH 7.2. A series of divers was then prepared, each containing a 1.5-cu. mm. sample of the mixture or the same volume of a preparation containing enzyme which had been heated in a boiling water bath for 5 to 10 minutes prior to mixing with cytochrome and ascorbic acid. In some cases divers were prepared so that comparisons of enzyme activity in the presence and absence of added cytochrome could be made. All experiments were conducted at 25°C.

Results.—1. Inhibition of Respiration by Cyanide and Azide.—The

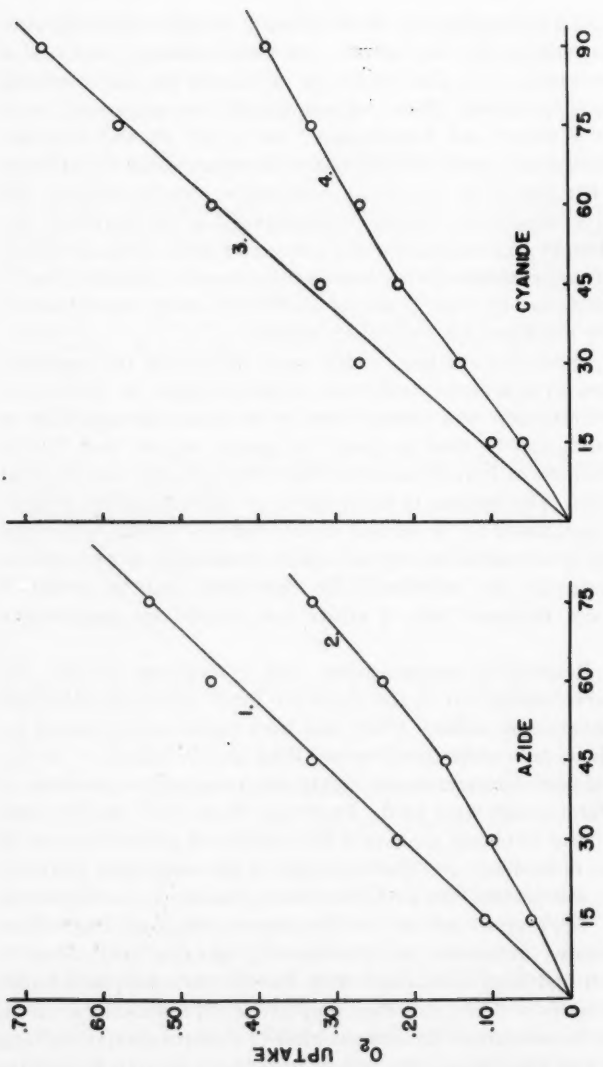

FIGURE 1

The effect of 10^{-2} molar sodium azide (Curve 2) and 10^{-2} molar KCN (Curve 4) on the respiration of *Paramecium calkinsi*. Curves 1 and 3 denote respiratory rates of untreated animals. The pH of the medium with azide was 6.2; that with cyanide, 7.0. Cyanide had been neutralized with HCl before use. An appropriate KCN-KOH mixture[23] was used to absorb carbon dioxide. The ordinate denotes oxygen uptake in mμl. per Paramecium. (One mμl. = 10^{-3} cu. mm.)

inhibition of respiration of intact cells by cyanide, azide, carbon monoxide, etc., has generally been interpreted as due to interference with the functioning of cytochrome and cytochrome oxidase. Thus, susceptibility to cyanide has been used as an indicator of the operation *in vivo* of this system of respiratory catalysts. The results of a typical experiment with these

inhibitors are shown graphically in figure 1. Curves 2 and 4 of this figure show, respectively, the effect of sodium azide and potassium cyanide, each in a final concentration of 10^{-2} molar, on the oxygen consumption of Paramecium. With both these substances respiration is depressed to approximately 50 per cent of the normal, and data are available which show that this inhibition is completely reversible. At acid pH values, however, Paramecia are irreversibly affected by strong concentrations of azide. Such animals apparently lose their ability to maintain water balance, for the contractile vacuoles stop beating and become enormously swollen.

In some experiments in which azide was used, there was a period of accelerated oxygen consumption following the inhibition of respiration. In Curve 2, representing only the first 75 minutes of a longer experiment, such a rise in respiratory rate can be seen. The rate of respiration stimulated by azide may, under certain experimental conditions, considerably exceed the normal, and it is of interest to note that the acceleration of oxygen consumption produced by azide is susceptible to cyanide.

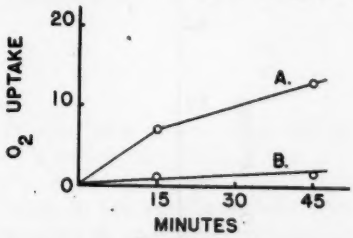

FIGURE 2

Oxidation of *p*-phenylenediamine by oxidase from *Paramecium calkinsi*. Experimental conditions: 25°C.; pH 7.2; final concentrations of cytochrome-c = 1×10^{-4} molar, of *p*-phenylenediamine = 0.033 molar. Oxidase preparation equivalent to approximately 75 Paramecia.

Curve *A* = oxidase + cytochrome + *p*-phenylenediamine; Curve *B* = same but with boiled oxidase. Ordinate denotes oxygen uptake in mμl.

The control respiratory rates, as shown by Curves 1 and 2 in figure 1, although identical, were obtained from two different samples of Paramecium and at different pH values, and, incidentally, it may be mentioned that the average respiratory rate for these animals is essentially identical with that reported for *P. calkinsi* of the same race and mating type by Boell and Woodruff.[15]

2. Cytochrome Oxidase Activity.—Preliminary tests of the cytochrome oxidase activity of Paramecium were performed with 0.033 molar *p*-phenylene diamine as substrate (Fig. 2). However, this compound, although it was readily oxidized, appeared to be toxic to the oxidase as evidenced by a rapid decline in the rate of O_2 uptake (cf. Schneider and Potter[16]). Its use was therefore abandoned. Ascorbic acid in a final concentration of 0.01 or 0.02 molar was used instead, since a number of workers had shown that it can reduce cytochrome-c and could, accordingly, be used as a substrate in tests for cytochrome oxidase.[16–19]

The results of a typical experiment are presented graphically in figure 3 and show unmistakably the presence of an enzyme in Paramecium capable

of oxidizing ascorbic acid. Moreover, since the catalytic oxidation of ascorbic acid is much greater in the presence of cytochrome than in its absence, it seems safe to conclude that the enzyme in this case is cytochrome oxidase. In this connection it may be recalled that Sato and Tamiya[20] reported finding the absorption bands of reduced cytochrome in Paramecium.

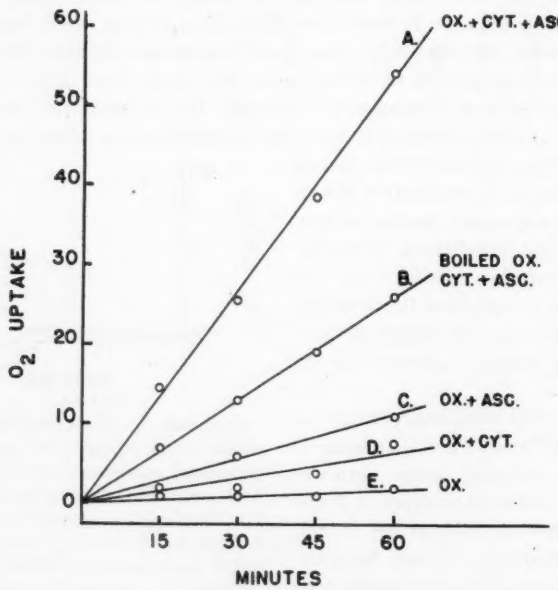

FIGURE 3

Oxidation of ascorbic acid by oxidase from *Paramecium calkinsi*. Experimental conditions: the same as stated for figure 2 except that ascorbic acid in a final concentration of 0.01 molar was used as substrate in place of *p*-phenylenediamine.

Curve *A* = oxidase + cytochrome + ascorbic acid; Curve *B* = same but with boiled oxidase; Curve *C* = oxidase + ascorbic acid; Curve *D* = oxidase + cytochrome; Curve *E* = oxidase. Ordinate denotes oxygen uptake in mμl.

Ascorbic acid in solution shows a fairly rapid oxygen uptake even in the absence of enzyme which has been shown to be due to the presence in the reaction mixture of metallic copper impurities.[19, 20] It is necessary, therefore, to run a blank containing boiled enzyme, cytochrome and substrate in order to determine the non-enzymic oxidation of ascorbic acid. However, since Stotz, *et al.*,[19] have shown that the catalytic effect of metallic copper is eliminated by the presence in the reaction mixture of undenatured

protein, as well as other organic compounds,[21] e.g., glycine,[22] it seems fairly certain that this method introduces an over-correction. Consequently the cytochrome oxidase values obtained are minimal.

Schneider and Potter[16] have described a method of correction which involves making determinations of the oxidase activity at three different concentrations and then extrapolating these to zero in order to determine the oxygen uptake due to non-enzymic catalysis. Such a procedure has been used in the preparation of table 1 which shows, in addition, a direct relationship between enzyme activity and enzyme concentration.

TABLE 1

NUMBER OF ANIMALS IN 1.5 CU. MM. OF REACTION MIXTURE	O_2 UPTAKE mμl/HOUR	CORRECTED CYTOCHROME OXIDASE ACTIVITY	RATIO: ACTIVITY/NUMBER
0	35*	0	..
102	57	22	21
204	76	41	20
306	96	61	20

* Derived by extrapolating the O_2 uptake at other values to zero enzyme concentration.[16]

Summary.—By a micromanometric method, the respiration of *Paramecium calkinsi* has been found to be sensitive to azide and cyanide. In addition, an enzyme capable of oxidizing ascorbic acid and *p*-phenylenediamine in the presence of cytochrome-c and which may, therefore, be designated as cytochrome oxidase has been demonstrated.

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*OFFSPRING FROM UNBORN MOTHERS**

BY W. L. RUSSELL AHD PATRICIA M. DOUGLASS

ROSCOE B. JACKSON MEMORIAL LABORATORY, BAR HARBOR, MAINE

Communicated October 29, 1945

The preliminary experiments reported here show that mouse ovaries transplanted from embryos to adult females can develop to maturity and produce offspring.

Eight such operations were performed, three of which have proved successful. The donor ovaries were obtained from embryos aged $13^1/_2$ days (in 3 of the operations) and $14^1/_2$ days (5 operations) post coitus, matings being timed by the vaginal plug method. They were transplanted to the ovarian capsules of young adult animals whose ovaries had been removed immediately before. A male was placed with each host a few days after the operation. Other transplantation experiments have shown that ovarian regeneration frequently occurs in host animals.[1] In order to distinguish offspring of the transplanted ovaries from offspring of regenerated ovaries a mating plan was used which has been described by Russell and Hurst.[1] This plan uses an inbred strain, stock 129, which carries both chinchilla, c^{ch}, and albino, c^a, genes. The embryo donors were obtained from matings of stock 129 albinos and were, therefore, of the genotype c^ac^a. The hosts were hybrids, although for the purpose of this experiment, transplantations could have been made within the 129 stock. The hybrids were the offspring of matings of 129 $c^{ch}c^{ch}$ × C57 black strain and, therefore, had the genetic constitution Cc^{ch}. These Cc^{ch} hosts with c^ac^a ovary implants were mated with 129 $c^{ch}c^{ch}$ males. Offspring of regenerated host ovaries would, therefore, be either Cc^{ch} or $c^{ch}c^{ch}$, while offspring from the transplanted ovaries would be $c^{ch}c^a$.

The animals used have not yet reached the end of their breeding period, but the three successful operations have so far yielded, respectively, 3, 4 and 10 offspring from the transplanted ovaries. In these operations the donor ovaries were obtained from embryos aged $14^1/_2$ days pc and were transplanted to hosts 40 days old. Regeneration of host ovaries occurred to some extent in all three cases as well as in four of the unsuccessful operations. The remaining unsuccessful operation produced no offspring.

The demonstration that offspring can be obtained from transplanted embryonic ovaries, that is, from unborn mothers, introduces a method that should prove of value in several different fields of experimental zoölogy. Some of the possible uses are discussed below.

1. Several dominant gene mutations apparently produce, in the homozygous mutant type, a lethal condition causing death of the animal while still within the uterus of the mother. The evidence rests on the proportion and nature of the abnormal embryos produced by matings of the heterozygotes. Transplantation of ovaries of the lethal type should, first of all, prove whether the ovary can survive in a normal host and, second, if the ovary does function, it would make possible a direct breeding test of the genetics of the lethal type itself. One experiment of this kind is being made by the authors.

2. In the study of chromosome translocations, experiments similar to the above could be undertaken to determine the chromosome make-up of embryos that die presumably from chromosome unbalance.

3. Ovarian transplantation could be used to identify the genetic type of embryos that have to be killed for study. An example will perhaps make this clearer. Mice homozygous for the gene *W* die shortly after birth from severe anemia and can, therefore, be obtained only from matings of the heterozygotes. Grüneberg[2] points out that since the anemia of *WW* embryos is already well marked on the 16th day it must have started earlier. Its actual onset has not been determined because there was no way of identifying the homozygous segregants before that age.

4. Many transplantation experiments that were possible with older ovaries could employ embryonic ovaries to obtain additional information. For example, in studies of differences in maternal environment the foster mother's influence could be brought into play at an earlier stage in the development of the ova.

5. The relation of hormones and other factors to the development and functioning of the ovary could be investigated by transplanting embryonic ovaries to hosts of various ages or to hosts subjected to various experimental treatments.

6. It is possible that transplantation would prove successful with still earlier embryonic stages. In that case it might be a useful technique for investigations on the origin of the germ cells.

7. There is no reason to suppose that the method could not be repeated for any number of successive generations, thus leading to an indefinite number of unborn direct female ancestors. This possibility should be of interest to investigators in several fields of research. In studies of the mammary tumor agent, for example, mice could be obtained whose female ancestors, for any number of generations, had not been nursed.

Summary.—Offspring were obtained from transplanted embryonic

mouse ovaries. This introduces a method that could be used for the genetic identification of some lethals and certain types of embryos that have to be killed for examination. It should prove of value in studies on differences in maternal environments, in experiments on the relation of hormonal and other factors to the development and functioning of the ovary and possibly for investigations on the origin of the germ cells. An extension of the method over successive generations, to produce animals descended from any number of unborn direct female ancestors, would provide still further possibilities for research.

* Aided by a grant from the Rockefeller Foundation and by grants to the Roscoe B. Jackson Memorial Laboratory from the National Cancer Institute, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the International Cancer Research Foundation.

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*INDUCTION OF CONJUGATION IN PARAMECIUM BURSARIA AMONG ANIMALS OF ONE MATING TYPE BY FLUID FROM ANOTHER MATING TYPE**

BY TZE-TUAN CHEN

DEPARTMENT OF ZOÖLOGY, UNIVERSITY OF CALIFORNIA, LOS ANGELES

Communicated October 25, 1945

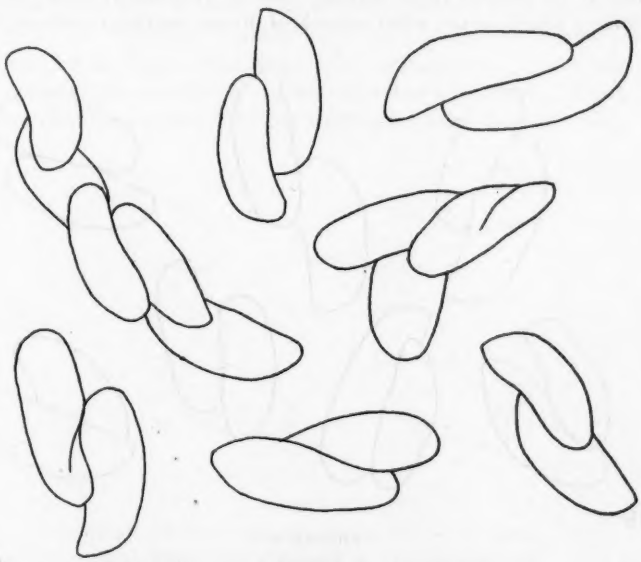
In these experiments, it was found that animal-free fluid from a Russian clone (Ru22) induces clotting and conjugation among animals of another mating type, even though the latter belongs to a different variety.[1]

Most of the present work was done on the effects of fluid from cultures of a Russian clone (Ru22) on animals of mating types belonging to four other varieties. This Russian clone (together with a number of other Russian clones) was sent to the present writer from Moscow by Professor G. F. Gause. Jennings and Opitz[2] found that this clone did not mate with any of the four varieties known at that time and they were of the opinion that this clone possibly belongs to a fifth variety. In the present paper it is called the fifth variety. (Apparently only one mating type of this variety has been found.)

The animals of this Russian clone (as well as those of other clones referred to in this paper) were cultured in essentially the manner described by Jennings.[3]

In testing the effect of fluid from the Russian clone on animals of another mating type, the fluid is first taken out of the culture (Ru22) with a micro-

pipette; it is then placed in depression slides and carefully examined under a dissecting microscope. All the animals, if any are accidentally included in the fluid, are removed. Then a number of animals of the clone to be tested, together with a very small quantity of fluid from this clone, are added to the Ru22 fluid. The depression slides are then left in a moist chamber and examined from time to time. As a control, fluid from the clone to be tested is first taken out of the culture, placed in a depression slide, and then animals from the same culture (or another culture of the same clone) are added.



1a

FIGURE 1a

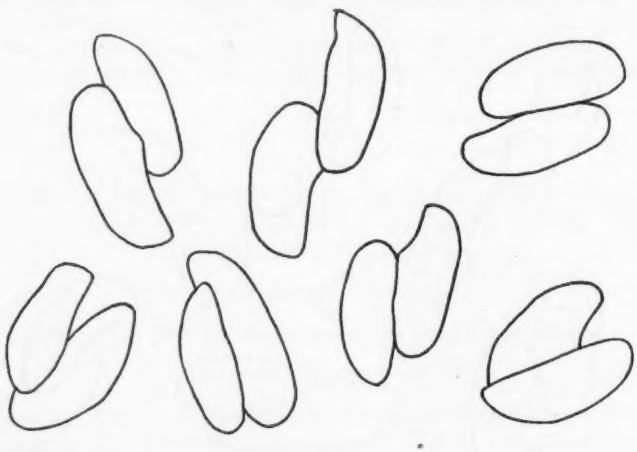
Clotting and pair-formation among animals of an English clone (En1) induced by fluid from a Russian clone (Ru22).

The fluid from cultures of the Russian clone (Ru22) was tested on animals of a number of mating types belonging to four other varieties (II, III, IV, VI) and especially on animals of an English clone (En1) belonging to the sixth variety.

Effects of the Ru22 Fluid on Animals of the English Clone (En1).—This English clone (En1) was collected by Professor E. G. Pringsheim in Cambridge, England, and was sent by him to the present writer, who subsequently made numerous tests on it. The results so far obtained show that it will not mate with animals of any of the known varieties but will con-

jugate readily with animals of a clone collected from Prague, Czechoslovakia (also sent by Professor Pringsheim). It seems clear that this English clone together with the Czechoslovakian clone constitute a new variety, which is designated as Variety VI.

Clotting: The animal-free fluid from Ru22 cultures apparently renders the surface of the animals of the English clone sticky so that the animals adhere to one another, forming clots and pairs (Fig. 1*a*). No such clotting or pair-formation was ever observed in the controls. The clotting or group-formation of animals of the English clone (one mating type)[4] induced by the fluid of the Russian clone (another mating type) differs somewhat from the clotting which occurs when animals of diverse mating types are mixed.



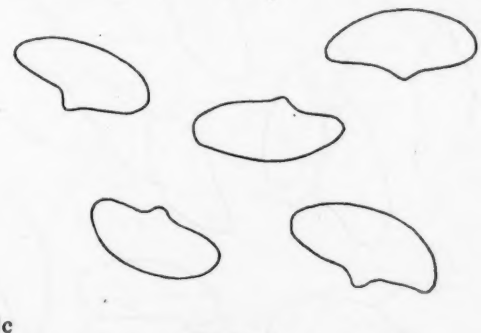
1b

FIGURE 1*b*

Pairs formed among animals of an English clone (En1) induced by the fluid from a Russian clone (Ru22).

The difference is twofold: (1) The clotting among animals of the same mating type occurs much later than that which usually occurs when animals of diverse mating types are mixed. In the former case, agglutination occurs 4–6 (usually more) hours after the animals of one mating type are introduced into the fluid of another mating type; in the latter case, agglutination occurs immediately or almost immediately. (2) The clots formed by animals of the same mating type are much smaller than those formed as a result of mixing animals of diverse mating types. In induced group-formation, each clot usually consists of 2–7 individuals. Clots formed as a result of mixing animals of diverse mating types are usually larger; each of the larger clots often consists of many individuals.

Conjugation: Pair-formation among animals of the same mating type[5] differs somewhat from pair-formation when those of diverse mating types are mixed. In the first place, in induced conjugation, the pairs are formed much later (4–6 or more hours after the animals are introduced into the fluid of another mating type). When animals of diverse mating types are mixed, usually many pairs are formed in one or two hours. Secondly, in induced conjugation many of the pairs are atypical. Although some of the pairs are hardly distinguishable from those formed as a result of mixing animals of diverse mating types, other pairs are atypical in that the two conjugants do not have the relative positions typical of normal conjugating pairs (Fig. 1*b*). Thirdly, in many pairs, the conjugants are not held as firmly together as those formed in ordinary conjugation. As a result, in a number of cases the conjugants become separated when fixed. This is true even after the animals have been conjugating for more than 20 hours.



1c

FIGURE 1*c*

Single animals of an English clone (En1) undergoing perhaps autogamy or endomixis or some other type of nuclear processes induced by the fluid from a Russian clone (Ru22).

Fixed and stained preparations of induced pairs show that nuclear changes occur in the conjugants and anlagen are formed in the ex-conjugants. The nuclear changes in induced conjugation are being studied and will be reported on separately.

Induction of Nuclear Changes in Single Animals: The Ru22 fluid induces not only clotting and conjugation, as already described, but also nuclear changes in some individuals not associated with any others. These single animals develop a paroral cone (Fig. 1*c*). This structure has been reported in *Paramecium aurelia* undergoing autogamy (endomixis).[6] Preliminary cytological studies show that nuclear changes take place in these single animals. It remains to be determined whether they undergo autog-

amy, or endomixis, or some other type of nuclear processes. These single animals may be found together with the conjugating pairs; at other times, however, they may be found alone.

In addition to the induction of clotting and conjugation among animals of the same mating type and of nuclear changes in some single animals, there are other effects of the Ru22 fluid on the members of the English clone. These additional effects are recognizable even before clotting and conjugation occur. The animals become (1) sluggish in movement, (2) darker in color, and (3) distorted in form to a greater or less extent. No such changes were found among the animals in the controls.

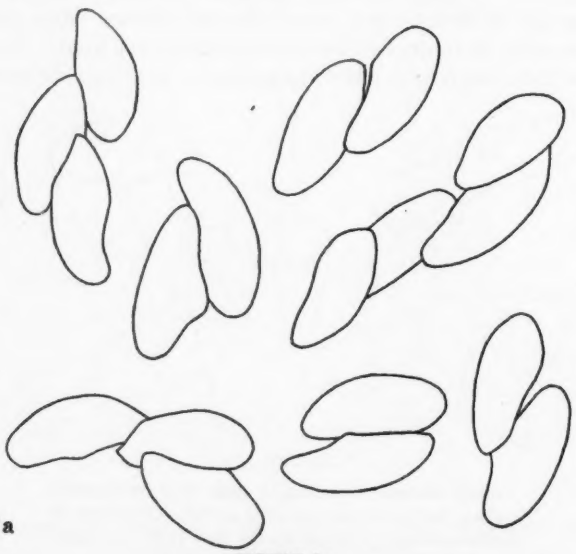


FIGURE 2a

Clotting and pair-formation among animals of an American clone, MM17 (Variety II), induced by fluid from a Russian clone (Ru22).

It should be noted that only a percentage (usually small) of the animals are visibly affected by the Ru22 fluid, and hence only a small number of them undergo clotting and conjugation; while a few solitary animals undergo nuclear changes. It is difficult to understand why only a percentage of animals are obviously affected. It seems probable that there are differences in physiological condition even among the animals of the same culture. It thus seems that those individuals which are affected differ from others added to the fluid at the same time.

Effect of Washing Animals.—In order to determine whether or not induction of clotting and pair-formation among members of the English clone En1 by the Ru22 fluid might be due to some impurities in the cultures of the Russian clone, on November 23, 1944, a number of the animals of this Russian clone were carefully washed and then recultured. Some weeks later the fluid was tested again on the animals belonging to the English clone En1. Clotting and pair-formation were again observed, as described above.

Effects of the Ru22 Fluid on Animals Belonging to Some Additional Varieties.—The fluid of the Russian clone Ru22 is apparently effective in inducing conjugation throughout the species since it induces conjugation in all the four varieties so far tested. The one other variety (I) is being tested.

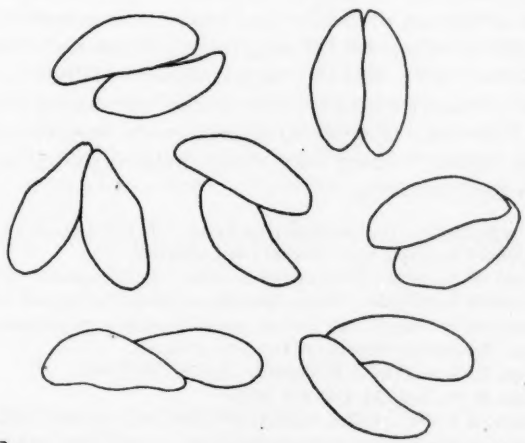


FIGURE 2*b*

Pairs formed among animals of an American clone, MM17 (Variety II), induced by the fluid from a Russian clone (Ru22).

In addition to inducing conjugation among the animals of the English clone (En1) belonging to the sixth variety, this Ru22 fluid also induces clotting and conjugation in each of the following clones: MM17 (mating type F) and Gr13 (type K) of the second variety, clones Gr1 (type N) and Pi3 (type O) of the third variety, and clone Ru3 (type R) of the fourth variety.[7] Clotting and pair-formation in these varieties are essentially the same as those which occur among animals of the English clone as described above (cf. Figs. 1*a*, 1*b* and 2*a*, 2*b*). No clotting or conjugation was ever observed in the controls. Some other clones of these three varieties were

also tested but as yet no clotting or conjugation has been observed. These negative results might be due to differential effects of the Ru22 fluid on different clones of the same variety.

Discussion.—The phenomena in *P. bursaria* described in this paper are possibly related to those of "sex stuffs" found in certain algae (Geitler,[8] Moewus[9]) and Protozoa (Kimball[10]). In *Tetraspora lubrica* Geitler found that centrifugates of culture fluid of one sex cause atypical group-formation among cells of the other sex. Copulation, however, does not follow this type of group-formation. In *Chlamydomonas eugametos* Moewus discovered the same effect of filtrates or centrifugates of culture fluid of one sex on individuals of the other sex. In *Euplotes patella* Kimball found that conjugation can be induced between animals of the same mating type by placing them in fluid in which another mating type has been living.

Another possibility is that the phenomena in *P. bursaria* described in this paper are similar to those of the "killers" as reported by Sonneborn[11] for *P. aurelia* in view of the following facts: (1) The Ru22 fluid makes the animals of the English clone En1 sluggish, darker and distorted in shape and these phenomena are similar to those in lethal interactions between diverse stocks in *P. aurelia*. (2) In *P. aurelia* Sonneborn (unpublished) found that one of the "killers," among other effects, induced pairing as described in this paper for *P. bursaria*.

* Aided by grants from the Committee for Research in Problems of Sex of the National Research Council and from the University of California.

[1] The fluid of two other Russian clones (Ru21, Ru30) appears to have the same effects as those of Ru22 fluid. These three clones belong to the same mating type and are characterized by a large body and an unusually large micronucleus. These clones are vigorous; flourishing cultures can be easily obtained.

[2] Jennings, H. S., and Opitz, P., *Genetics*, **29**, 576–583 (1944).

[3] Jennings, H. S., *Ibid.*, **24**, 202–233 (1939).

[4] Of course, it is also possible, though not probable, that the Ru22 fluid causes a change in mating type in some of the animals of the English clone, and hence in this case it is merely a conjugation between animals of diverse mating types.

[5] In addition to the conjugating pairs, some "threes" were also observed. (A group of three animals in conjugation is to be called a "three.") In each "three," nuclear changes occur in all three conjugants and anlagen are formed in the ex-conjugants.

[6] Diller, W. F., *Jour. Morph.*, **59**, 11–67 (1936); Caldwell, L., *Jour. Exper. Zool.*, **66**, 371–407 (1933).

[7] As yet only a few clones of these three varieties have been tested. It is likely that induced conjugation will be found in more clones when a greater number of clones are tested.

[8] Geitler, L., *Biol. Zentralbl.*, **51**, 173–187 (1931).

[9] Moewus, F., *Arch. Protistenk.*, **80**, 469–526 (1933).

[10] Kimball, R. F., *Genetics*, **27**, 269–285 (1942).

[11] Sonneborn, T. M., *Amer. Nat.*, **73**, 390–413 (1939).

MARKOFF CHAINS WITH REVERSE TRANSITIONS

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Consider a system which may take $n + 1$ states: $0, 1, \ldots, n$. Let the probability of transitions $(i - 1 \to i)$ and $(i + 1 \to i)$ during any time dt be, respectively, $k_i dt + o(dt)$ and $g_i dt + o(dt)$. Let these be the only transitions possible during dt and let the system be in the state 0 at $t = 0$. If $Y_i(t)$ is the probability that the system be in the state i at the time t, then $Y_0(0) = 1$, $Y_i(0) = 0$ for $i \geqslant 1$ and[1]

$$dY_i/dt = k_i Y_{i-1} - k_{i+1} Y_i + g_i Y_{i+1} - g_{i-1} Y_i, \tag{1}$$

where by definition $Y_{n+1} = Y_j = g_j = 0$ if $j < 0$. In certain biophysical applications[2] of (1) the only function of interest is $Y_n(t)$ and $k_i = k$ or $= 0$, $g_i = g$ or $= 0$, where k, g are constants > 0. The purpose of this paper is to derive, under these assumptions, an explicit expression of $Y_n(t)$. The method used is that of Laplace transformation which has been introduced into this field by H. Bateman.[3] This transformation seems to be essential here because it shows an intimate connection of our problem with *Tchebychef polynomials*.

Put $y_i(s) = \int_0^\infty e^{-st} Y_i(t) dt$ and consider first the following three processes.

First Process.—Let $k_i = k (1 \leq i \leq n)$, $g_i = g (0 \leq i \leq n - 1)$, $k_{n+1} = 0$. Applying the Laplace transformation to (1) we obtain a system of ordinary linear equations in y_i's, which gives

$$y_n(s) = k^n/[E_{n+1} - (k + g)E_n + kgE_{n-1}], \tag{2}$$

E_n being a continuant, i.e., a determinant $\|a_{i,j}\|_n$ with

$$a_{i,i} = s + k + g,\ a_{i,i+1} = g,\ a_{i-1,i} = k,\ a_{i,j} = 0 \text{ for } |j - i| > 1. \tag{3}$$

Expanding E_{n+1} according to the elements of the last column we have

$$E_{n+1} = (s + k + g)E_n - kgE_{n-1}. \tag{4}$$

By (2) and (4) we obtain

$$y_n(s) = k^n/(sE_n). \tag{5}$$

Solving (4) as a finite difference equation in E_n we have[4]

$$E_n = [(kg)^n/(\sigma^2 - 1)]^{1/2}[(\sigma + \sqrt{\sigma^2 - 1})^{n+1} - (\sigma - \sqrt{\sigma^2 - 1})^{n+1}]/2,$$

where

$$\sigma = (s + k + g)/[2(kg)^{1/2}] = \cos\theta. \tag{6}$$

But arccosh $\sigma = i\theta = \log_e(\sigma + \sqrt{\sigma^2 - 1}) = -\log_e(\sigma - \sqrt{\sigma^2 - 1})$. Therefore,

$$E_n = (kg)^{n/2} \sin(n\theta + \theta)/\sin\theta. \tag{7}$$

The factor in θ is here a standard form of the Tchebychef polynomial of 2nd kind.[5] If $|\sigma| > 1$ we take $\sigma = \cosh\theta$ and replace in (7) sin by sinh. By (7), $E_n = 0$ for

$$\sigma = \cos(i\pi\nu) \text{ where } \nu = 1/(n+1),\ i = 1, 2, \ldots, n. \tag{8}$$

Therefore, E_n considered as a polynomial in s has its n roots[6] $s = -\bar{k}_i$, where

$$\bar{k}_i = k + g - 2(kg)^{1/2}\cos(i\pi\nu) > 0. \tag{9}$$

By (3) the highest term of E_n in s is s^n. Therefore

$$E_n = \Pi_{i=1}^{i=n}(s + \bar{k}_i). \tag{10}$$

Consequently by (5) and by known theorems of Laplace transformation:[1, 8]

$$Y_n(t)/k^n = 1^* \exp(-\bar{k}_1 t)^* \exp(-\bar{k}_2 t)^* \ldots {}^* \exp(-\bar{k}_n t) = \tag{11}$$

$$(\Pi_{i=1}^{i=n}\bar{k}_i)^{-1} - \textstyle\sum_{i=1}^{i=n}[\bar{k}_i \Pi_{j=1, j\neq i}^{j=n}(\bar{k}_j - \bar{k}_i)^{-1}\exp(-\bar{k}_i t), \tag{12}$$

$$Y_n(t) = P - (2\sqrt{g/k})^{1-n} e^{-(k+g)t} \textstyle\sum_{i=1}^{i=n} H_i e^{\tau_i t}/c_i, \tag{13}$$

where * stays for convolution, $1^* \ldots = \int_0^t \ldots dt$ and

$$c_i = 1 + (g/k) - 2(g/k)^{1/2}\cos(i\pi\nu),\ 1/P = \Pi_{i=1}^{i=n} c_i, \tag{14}$$

$$1/H_i = \Pi_{j=1, j\neq i}^{j=n}[\cos(i\pi\nu) - \cos(j\pi\nu)],\ \tau_i = 2t\sqrt{kg}\cos(i\pi\nu). \tag{15}$$

From (14) we have by a known formula[7]

$$P = [1 - (g/k)]/[1 - (g/k)^{n+1}]. \tag{14'}$$

From (13) we see that $Y_n(t) \to P$ when $t \to \infty$, because $(k+g)t - \tau_i = \bar{k}_i t > 0$. A simple formula for H_i is obtained as follows: from (7) (9) (10) and (6) we have

$$\Pi_{j=1}^{j=n}[\cos\theta - \cos(j\pi\nu)] = 2^{-n}\sin(n\theta + \theta)/\sin\theta, \tag{16}$$

which could be derived also from known relations for trigonometric products.[7] Putting $\theta = i\pi\nu + \delta$ in (16) we see from (15) that H_i is the limit for $\delta = 0$ of

$$[\cos(i\pi\nu + \delta) - \cos(i\pi\nu)]/\Pi_{j=1}^{j=n}[\cos(i\pi\nu + \delta) - \cos(j\pi\nu)] = 2^n[\ldots]\sin(i\pi\nu + \delta)/\sin[(n+1)(i\pi\nu + \delta)],$$

where $[\ldots]$ is in both numerators the same. Consequently

$$H_i = -(-1)^i 2^n \nu \sin^2(i\pi\nu).$$

Second Process.—Let $k_i = k(1 \leq i \leq n)$, $g_i = g\ (0 \leq i \leq n-2)$, $k_{n+1} = g_{n-1} = 0$. We have now

$$y_n(s) = k^n/(sG_n) \text{ where } G_n = E_n - gE_{n-1}. \tag{17}$$

$Y_n(t)$ is still given by (11) (12) if the roots of G_n are called $s = -\bar{k}_i$ $(i = 1, \ldots, n)$. Since[2] $(E_n)_{s=0} = k^n/P$ by (10), (9), (14), from (14′) we have $(G_n)_{s=0} = k^n$. Therefore:

$$\bar{k}_1\bar{k}_2 \ldots \bar{k}_n = k^n, \tag{18}$$

which reduces by one the number of roots to be calculated. We write:

$$\bar{k}_i = k + g - 2(kg)^{1/2} \cos\theta_i, \ (i = 1, \ldots, n), \tag{19}$$

where the θ_i's are n values of θ[cf. (6)]. We locate these values so that the calculation of the roots may be carried out by routine methods. From (7) (17) we have

$$G_n = -(-1)^i(kg)^{n/2} \text{ for } \theta = (i-1)\pi/n, \ (i \geq 2), \tag{20}$$

$$G_n = (-1)^i(kg)^{n/2}\,(g/k)^{1/2} \text{ for } \theta = i\pi/(n+1), \ (i \geq 1), \tag{21}$$

$$G_n = (kg)^{n/2}\,[n + 1 - n(g/k)^{1/2}] \text{ for } \theta = 0. \tag{22}$$

Therefore, if $g/k < (1 + n^{-1})^2$, so that $[\ldots]$ in (22) is > 0 we have:

$$(i-1)\pi/n < \theta_i < i\pi/(n+1), \qquad (i = 1, \ldots n), \tag{23}$$

which gives a particularly good location of the n roots for large n. If $[\ldots]$ in (22) is < 0, we must take (23) for $2 \leq i \leq n$, which locates only $n - 1$ roots, however, the nth root may be calculated from (18).[3] From (18) and (12) we see that $Y_n(t) \to 1$ as $t \to \infty$.

Third Process.—Let $k_i = k(1 \leq i \leq n+1)$, $g_i = g(0 \leq i \leq n-1)$. We have now $y_n(s) = k^n/G_{n+1}$. The $n + 1$ roots of G_{n+1} are located by equations (18) to (23) in which, however, n must be changed into $n + 1$. By known methods[1, 3] we have now:

$$\begin{aligned} Y_n(t) &= k^n \exp(-\bar{k}_1 t) * \exp(-\bar{k}_2 t) * \ldots * \exp(-\bar{k}_{n+1} t) \\ &= k^n \textstyle\sum_{i=1}^{n+1} [\Pi_{j=1,\, j\neq i}^{n+1} (\bar{k}_j - \bar{k}_i)]^{-1} \exp(-\bar{k}_i t). \end{aligned}$$

Since $\bar{k}_i > 0$, we have $Y_n(t) \to 0$ as $t \to \infty$.

More General Processes.—If in the previous processes some g_i's with $i \leq n - 2$ are zero, it is easy to see[2] that the corresponding y_n is a product of some y_n's of the previous three types, therefore, the $Y_n(t)$ is a convolution of some Y_n's of the same types.

[1] For bibliography on the subject cf., e.g., Opatowski, I., these PROCEEDINGS, 28, 84–88 (1942). The probabilities $k_i dt + o(dt)$ and $g_i dt + o(dt)$ are conditional; they give the probabilities of transitions within the interval of time $(t, t+dt)$ under the assumption that at the time t the states $i-1$ and $i+1$ existed, respectively.

[2] Opatowski, I., *Bull. Math. Biophysics*, **7**, 161–180 (1945); **8** (1946).
[3] Bateman, H., *Proc. Phil. Soc. Cambridge*, **15**, 423–427 (1910).
[4] Scott, R. F., *Messenger of Math.*, **8**, 131–138 (1879).
[5] Szegö, G., *Orthogonal Polynomials*, New York, 1939, pp. 3, 118.
[6] Cf. Muir, T., *Educational Times*, **42**, 95–96 (1885).
[7] Cauchy, A. L., *Algebraische Analyse*, Berlin, 1885, pp. 381–382.
[8] Instead of (18) the relation $\Sigma_{i=1}^{i=n} k_i = nk + (n-1)g$ can also be used to calculate one of the roots when all the remaining roots are known.

GENERALIZED ARC-SETS

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The purpose of this note is to describe a type of strengthened extension set. These sets may be regarded as generalizations of the A-sets introduced by W. L. Ayres and G. T. Whyburn and as homotopic analogs of sets studied by the latter in his work on cyclic elements of higher order.

In what follows H will denote a compact (= bi-compact) Hausdorff space and *subspace* will mean *closed subset of* H. As usual S_n is the unit sphere of $(n+1)$-dimensional euclidean space. A subspace is a C_n if all mappings of it into S_n are inessential (and hence null-homotopic). By a T_n is meant a subspace all of whose closed subsets are of type C_n. Thus, in the sense of homotopy, a T_n is trivial in dimension n, at least in so far as spheres are concerned. Since an AR can be contracted over itself to a point it must be a C_n for all n. A p-cell is a T_n for $p \leq n$. But S_n is not a C_n and hence not a T_n.

If M is a subspace then M is termed an *extension set in dimension n* (or a J_n) provided that for each subspace X and mapping $f: M \cdot X \to S_n$ there is an $\bar{f}$; $X \to S_n$ such that $f = \bar{f} | M \cdot X$. A subset of H is a B_n if it is not cut by any T_n and is maximal relative to this property. This last concept is due to G. T. Whyburn. The sets T_n are homotopic analogs of sets used by Whyburn to define cyclic elements of higher order in the same manner in which the sets B_n are defined. It is to be emphasized that extension sets are defined *relative* to H and the same remark applies to the sets B_n.

The sets J_n suffer from the serious defect that they may lie wholly within a set of type B_n. For example, let N be defined by the equations $(t \geq 0)\ x = e^{-t} + |\cos t|$, $y = \sin t$, let L denote the segment joining $(2, 0)$ to $(2, -3)$ and $(2, -3)$ to $(0, -3)$. Finally, if N' is the image of N in the Y-axis and C the circle $x^2 + y^2 = 1$, let H be the union of N, N', L, L' and

C. Then H is a B_0 and C is a J_0. This latter fact follows readily from Theorem 1 below.

Suppose that X is a subspace and f a mapping of X into S_n. Then a closed subset Y of H is an *essential membrane* for f if f can be extended to a mapping of any closed proper subset of Y into S_n but not to a mapping of Y into S_n. This concept is due to W. Hurewicz.

THEOREM I. *In order that a subspace M be a J_n it is n.a.s. that for any closed set X in M and mapping $f: X \to S_n$, each essential membrane for f be contained in M.*

To avoid the difficulty indicated in the example we define a set N to be an A_n provided that

(i) N is a J_n,

(ii) If X is a B_n and $X \cdot N$ is not a T_n then X is a subset of N.

THEOREM II. *The intersection of any family of A_n's is an A_n. Each B_n is an A_n.*

This result is well known if $n = 0$ and H is Peanian. Analogous results hold for sets involving the higher order cyclic elements. In the proof use is made of the fact that J_n's have the same intersection property and that each B_n is a J_n.

THEOREM III. *Let X be a subspace such that H-X is the union of a family of pairwise disjoint open sets the boundary of each of which is a T_n. Then X is an A_n.*

Again when $n = 0$ and H is Peanian this theorem and its converse are well-known results of the cyclic element theory. As to the converse we give an example. Let X be the set $x^2 + y^2 + z^2 = 2$ and U that part of the set $(x^2 + y^2 = 1, 0 \leq z \leq 3)$ which lies *outside* of X. Then, if $H = X + U$, $H - X = U$ cannot be expressed as the union of a collection of pairwise disjoint open sets (other than the family consisting of U itself) and $\bar{U} - U$ is an S_1 and hence certainly not a T_1. It is easy to see that X is a B_1 and hence a J_1. Here H is an ANR and so locally connected in all dimensions in the strongest possible manner.

The space H is said to be of type V provided that for any pair of closed sets A and B there is a decomposition of H into closed sets A' and B' such that $A \subset A'$, $B \subset B'$, and $A' \cdot B' \cdot (A + B) = A \cdot B$. If H is metric it is of type V but there are spaces of type V which are not metric. The exact position of this axiom in the hierarchy of separation postulates is not known. It is clearly stronger than normality.

THEOREM IV. *If H is of type V then each A_n is an A_{n+1}.*

The proof of this proposition makes use of fact that each T_n is a T_{n+1} and each J_n is a J_{n+1}.

It is clear that H is an A_n and that any T_m is an A_n if $m \leq n$. Also it may be shown that if X is a C_n and M an A_n then $M \cdot X$ is a C_n. It follows that if H is a C_n so also is each A_n and hence each B_n

To generalize the notion of an end-point we say that a subspace E is a T_n-end-element if each neighborhood of E contains a neighborhood whose boundary is a T_n. In this way a point is an end-point if and only if it is a T_0-end-point.

THEOREM V. *Each T_n-end-element is an A_n.*

By a prime $J_n(A_n)$ is meant a $J_n(A_n)$ no closed subset of which is a $J_n(A_n)$ without at the same time being a T_n.

LEMMA 1. *Let A and B be subspaces such that A,B and $A \cdot B$ are T_n's and $A + B$ is a C_n. Then $A + B$ is a T_n.*

This result is no longer valid if the assumption that $A + B$ be a C_n is deleted. In general it can only be said that the union of two T_n's is a T_{n+1}.

LEMMA 2. *If X is an A_n, Z a T_n and $X - Z = U + V$, $U|V$, then the sets $U + Z$ and $V + Z$ are A_n's.*

With the aid of these results it may be shown that

THEOREM VI. *If H is a C_n each prime A_n is a B_n and conversely.*

THEOREM VII. *Let X and Y be subspaces such that $X + Y$ and $X \cdot Y$ are A_n's. Then each of X and Y is an A_n.*

For a Peanian H the original definition of an A_0 was as follows: X is an A-set if it contains each simple continuous arc whose end-points it contains. If now Q_{n+1} is an $(n + 1)$-cell it is clear from Theorem I that an A_n has the property that it contains each Q_{n+1} whose homology boundary it contains. Thus, if in the second example, we add to H the set $(x^2 + y^2 = 1, z = 3)$ then X is no longer an A_1 since it contains the boundary of a Q_2 without containing the Q_2.

LEMMA 3. *If H is a T_n then $H \times (01)$ is a T_{n+1}.*

With the aid of this result and certain others which follow from it we can show that

THEOREM VIII. *If H is a C_n and not a T_n then H contains non-degenerate sets B_{n-1}.*

It should be possible to replace the hypothesis that H is a C_n by the assumption that H is C_{n-1}.

If the mapping f of X into S_n is essential but not essential on any closed proper subset of X then f is said to be *irreducibly essential* on X. It may be shown, for example, that if H admits an essential mapping onto S_n then this mapping is irreducibly essential on some subspace. Parenthetically we interpolate the following: Suppose that H admits an irreducibly essential mapping onto an n-sphere. One might be inclined to conjecture that there exists a subspace with the property that every one of its essential mappings into S_n is irreducibly essential. This need not be true even if H is a finite dimensional metric space (of course compact).

PROPOSITION. *If H admits an irreducibly essential mapping onto S_n then H is not cut by a T_{n-1}.*

As of the present writing the validity of this assertion can only be affirmed in certain highly restricted situations.[1]

[1] A detailed discussion of the sets B_n, C_n, J_n and T_n will appear in the *Trans. Am. Math. Soc.* where pertinent references to the work of Borsuk, Eilenberg, Hurewicz and Whyburn will be given.

TWO MEAN THEOREMS IN HILBERT SPACE

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The ergodic theory and the researches concerning the law of large numbers in the Theory of Probability have demonstrated the importance of the study of the asymptotic behavior of the arithmetical mean

$$\frac{x_1 + x_2 + \ldots + x_n}{n}$$

of a sequence $\{x_n\}$ of points in Hilbert space. In this note we shall give two mean theorems, each of which can be regarded as generalizing the following well-known theorem due to J. von Neumann:[1] For any unitary operator U in the Hilbert space $\mathfrak{H}$ and for any given point x in $\mathfrak{H}$, the arithmetical mean of the n first iterated images of x

$$\frac{Ux + U^2x + \ldots + U^nx}{n}$$

converges strongly to a limit point.

THEOREM I. *Let $\{x_n\}$ be a sequence of points in Hilbert space (real or complex). If there are two constants c_1, c_2 such that the inequalities*

$$||x_{n+1} + x_{n+2} + \ldots + x_{n+m}||^2 - ||x_1 + x_2 + \ldots + x_m||^2 < c_1 m \quad (1)$$

and

$$||x_1 + x_2 + \ldots + x_{n+2}||^2 - 2||x_1 + x_2 + \ldots + x_{n+1}||^2 + ||x_1 + x_2 + \ldots + x_n||^2 > c_2 \quad (2)$$

hold for all positive integers m, n, then the limit

$$\lim_{n\to\infty} \frac{x_1 + x_2 + \ldots + x_n}{n} \quad (3)$$

exists in the sense of strong convergence.

Proof.—We denote as usual by (x, y) the scalar product of two points x, y in $\mathfrak{H}$ and use the following notations:

$$s_n = x_1 + x_2 + \ldots + x_n,$$

$$((x, y)) = (x, y) + (y, x),$$

$$\alpha_k = \sum_{i=1}^{k} ((x_i, x_{k+1})),$$

$$\beta_n = \sum_{k=1}^{n-1} \alpha_k.$$

Obviously the numbers $((x, y))$, α_k, β_n are always real.

It is easy to see that (1) is equivalent to the inequality

$$\left\| \frac{s_n}{n} - \frac{s_{n+m}}{n+m} \right\|^2 < \frac{m}{n(n+m)} \left(\frac{||s_n||^2}{n} + \frac{||s_m||^2}{m} - \frac{||s_{n+m}||^2}{n+m} + c_1 \right),$$

which can be written

$$\left\| \frac{s_n}{n} - \frac{s_{n+m}}{n+m} \right\|^2 < \frac{m}{n(n+m)} \left(\frac{1}{n} \sum_{i=1}^{n} ||x_i||^2 + \frac{1}{m} \sum_{i=1}^{m} ||x_i||^2 - \frac{1}{n+m} \sum_{i=1}^{n+m} ||x_i||^2 + c_1 \right) + \frac{m}{n(n+m)} \left(\frac{\beta_n}{n} + \frac{\beta_m}{m} - \frac{\beta_{n+m}}{n+m} \right). \quad (4)$$

But the hypothesis (1) implies also the boundedness of $||x_i||^2$. Let c_3 be a constant such that

$$||x_i||^2 < c_3 \quad (i = 1, 2, 3, \ldots). \quad (5)$$

Then the expression in the first bracket on the right-hand side of (4) is less than $c_4 = 2c_3 + c_1$. Hence, we have

$$\left\| \frac{s_n}{n} - \frac{s_{n+m}}{n+m} \right\|^2 < \frac{c_4}{n} + \frac{m}{n(n+m)} \left(\frac{\beta_n}{n} + \frac{\beta_m}{m} - \frac{\beta_{n+m}}{n+m} \right)$$

or

$$\left\| \frac{s_n}{n} - \frac{s_{n+m}}{n+m} \right\|^2 < \frac{c_4}{n} + \frac{m}{n+m} \frac{\beta_n}{n^2} + \left(\frac{m}{n+m} \right)^2 \frac{\beta_m}{m^2} - \frac{m}{(n+m)^2} \frac{\beta_{n+m} - \beta_m}{n}. \quad (6)$$

Now, in virtue of the Schwarz inequality and (5), the numbers α_k/k are bounded. If we set

$$\lambda = \limsup_{k \to \infty} \frac{\alpha_k}{k}, \quad (7)$$

we have

$$\frac{\beta_n}{n^2} = \frac{1}{n^2}\sum_{k=1}^{n-1} k\cdot\frac{\alpha_k}{k} < \frac{\lambda}{2} + \epsilon(n), \tag{8}$$

$\epsilon(n)$ being a function of n suitably chosen with $\lim_{n\to\infty} \epsilon(n) = 0$.

Making use of the hypothesis (2) and (5), we can find a positive constant c_5 such that

$$\alpha_{k+1} > \alpha_k - c_5 \qquad (k = 1, 2, 3, \ldots). \tag{9}$$

Consequently we have

$$\beta_{n+m} - \beta_m = \sum_{k=m}^{m+n-1} \alpha_k > n\alpha_m - \frac{n(n-1)}{2} c_5 > n(\alpha_m - nc_5). \tag{10}$$

From (6), (8) and (10), it follows that

$$\left\|\frac{s_n}{n} - \frac{s_{n+m}}{n+m}\right\|^2 < \frac{c_4}{n} + \frac{m}{n+m}\left(\frac{\lambda}{2} + \epsilon(n)\right) + \left(\frac{m}{n+m}\right)^2\left(\frac{\lambda}{2} + \epsilon(m)\right) - \frac{m}{(n+m)^2}(\alpha_m - nc_5). \tag{11}$$

Similarly, if l is a positive integer not greater than n, we may write the inequality[2]

$$\left\|\frac{s_l}{l} - \frac{s_{n+m}}{n+m}\right\|^2 < \frac{c_4}{l} + \frac{n+m-l}{n+m}\left(\frac{\lambda}{2} + \epsilon(l)\right) + \left(\frac{n+m-l}{n+m}\right)^2\left(\frac{\lambda}{2} + \epsilon(n+m-l)\right) - \frac{n+m-l}{(n+m)^2}(\alpha_m - nc_5). \tag{12}$$

According to (11), (12) and

$$\left\|\frac{s_n}{n} - \frac{s_l}{l}\right\|^2 \leqq 2\left\|\frac{s_n}{n} - \frac{s_{n+m}}{n+m}\right\|^2 + 2\left\|\frac{s_l}{l} - \frac{s_{n+m}}{n+m}\right\|^2, \tag{13}$$

we obtain for $l \leqq n$:

$$\left\|\frac{s_n}{n} - \frac{s_l}{l}\right\|^2 < \frac{2c_4}{n} + \frac{2m}{n+m}\left(\frac{\lambda}{2} + \epsilon(n)\right) + 2\left(\frac{m}{n+m}\right)^2\left(\frac{\lambda}{2} + \epsilon(m)\right) - \frac{2m}{(n+m)^2}(\alpha_m - nc_5) + \frac{2c_4}{l} + \frac{2(n+m-l)}{n+m}\left(\frac{\lambda}{2} + \epsilon(l)\right) + 2\left(\frac{n+m-l}{n+m}\right)^2\left(\frac{\lambda}{2} + \epsilon(n+m-l)\right) - \frac{2(n+m-l)}{(n+m)^2}(\alpha_m - nc_5). \tag{14}$$

If now we keep n, l fixed and let $m \to \infty$ through a suitable sequence of integral values such that $(\alpha_m/m) \to \lambda$, (14) becomes

$$\left\| \frac{s_n}{n} - \frac{s_l}{l} \right\|^2 \leqq 2c_4 \left(\frac{1}{n} + \frac{1}{l} \right) + 2\epsilon(n) + 2\epsilon(l). \tag{15}$$

As this inequality is symmetric with respect to n, l, it remains true even if we cease to assume $l \leqq n$. This inequality proves the existence of the strong limit (3).

THEOREM II. *Let $\{x_n\}$ be a sequence of points in Hilbert space (real or complex). If there is a constant c_1 such that the inequality (1) is fulfilled for all positive integers m, n and if the limit*

$$\lim_{n \to \infty} \frac{||x_1 + x_2 + \ldots + x_{n+1}||^2 - ||x_1 + x_2 + \ldots + x_n||^2}{n} \tag{16}$$

exists, then the strong limit (3) exists also.

Proof.—Using the same notations as above, we have still the inequality (6), which is, in fact, a consequence of the hypothesis (1) alone. But, according to the present second hypothesis, we have this time, instead of (7) and (8):

$$\lambda = \lim_{k \to \infty} \frac{\alpha_k}{k} \tag{17}$$

and

$$\frac{\beta_n}{n^2} = \frac{\lambda}{2} + \epsilon(n) \tag{18}$$

with $\lim_{n \to \infty} \epsilon(n) = 0$.

By setting

$$\gamma(n, m) = \frac{m}{(n+m)^2} \frac{\beta_{n+m} - \beta_m}{n}, \tag{19}$$

we infer from (17) that

$$\lim_{m \to \infty} \gamma(n, m) = \lambda \tag{20}$$

for any fixed value of n.

According to (18), (19), the inequality (6) may be written

$$\left\| \frac{s_n}{n} - \frac{s_{n+m}}{n+m} \right\|^2 < \frac{c_4}{n} + \frac{m}{n+m} \left(\frac{\lambda}{2} + \epsilon(n) \right) + \left(\frac{m}{n+m} \right)^2 \left(\frac{\lambda}{2} + \epsilon(m) \right) - \gamma(n, m). \tag{21}$$

Similarly, for any positive integer $l \leqq m$, we have

$$\left\| \frac{s_l}{l} - \frac{s_{n+m}}{n+m} \right\|^2 < \frac{c_4}{l} + \frac{n+m-l}{n+m}\left(\frac{\lambda}{2} + \epsilon(l)\right) + \left(\frac{n+m-l}{n+m}\right)^2 \left(\frac{\lambda}{2} + \epsilon(n+m-l)\right) - \gamma(l, n+m-l). \quad (22)$$

Now, using the inequality (13), we can deduce from (21), (22) an inequality which is like (14) and which can be again reduced to (15) by passing to the limit $m \to \infty$. Our theorem II is thus proved.

We terminate this note with the following remark from the point of view of the Theory of Probability. If we consider a sequence of chance variables instead of a sequence of points in Hilbert space, a similar argument will furnish two theorems giving new sufficient conditions for the validity of the law of large numbers. The results thus obtained contain as a particular case an important theorem due to A. Khintchine,[3] which asserts that any stationary sequence of chance variables obeys the law of large numbers.

[1] Neumann, J. von, these PROCEEDINGS, **18**, 70–82 (1932). See also Hopf, E., *Ergodentheorie*, Berlin, 1937, p. 23.

[2] We use here the inequality $\alpha_{n+m-l} - lc_5 \geqq \alpha_m - nc_5$, which follows readily from (9) and $l \leqq n$.

[3] Khintchine, A., *Rec. Math. Moscow*, **40**, 124–128 (1933).

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